T113
Y12
3933

YALE MEDICAL LIBRARY
3 9002 08627 9255

THE EFFECTS OF A RETINOBLASTOMA DERIVED GROWTH FACTOR ON THE HEALING OF EXPERIMENTAL WOUNDS IN THE RABBIT LENS AND RETINA

Eduardo Alfonso

1980

YALE

MEDICAL LIBRARY

Digitized by the Internet Archive
in 2017 with funding from
Arcadia Fund

https://archive.org/details/effectsofretinob00alfo

The Effects of a Retinoblastoma Derived Growth Factor on the Healing of Experimental Wounds in the Rabbit Lens and Retina

Eduardo Alfonso

B.A., Yale University, 1976

A Thesis Submitted to the Yale University School
of Medicine in Partial Fulfillment of the Requirement
for the degree of Doctor of Medicine

May, 1980

Med. Lib.

T113
Y120
3933

This work is dedicated to my family.

Acknowledgments

I am very grateful to Dr. Caleb Gonzalez for the interest and enthusiasm he has stirred in me as I begin my career in ophthalmology and to Dr. Ted Reid for his friendship, encouragement, constant supervision and support. I would also like to thank for thier suggestions and critical analysis of this work to Dr. Marvin L. Sears, Dr. James Puklin, Dr. Yin Lok Lai, and Dr. Daniel Albert. Technical parts of this work would have been impossible without the assistance of Alden Mead, Sue Forster, Norman Rubin, Ray Lug, Bill Caster, and Gary Fletcher. Photography assistance was provided by Bill McVerry and Bob Brown. Typing of the manuscript was expertly done by Stephanie Crowley.

TABLE OF CONTENTS

Introduction-- 1

I. Review of the Literature-------------------- 3
    A. Wounds in the retina------------------- 3
    B. Wounds in the lens----------------------- 12
    C. Growth factors--------------------------- 15
        1. Nerve growth factor (NGF)----------- 16
        2. Retinoblastoma derived growth factor (RDGF)----------------------- 18

II. Materials and Methods----------------------- 21
    A. Experimental animal--------------------- 21
    B. Anesthesia------------------------------- 24
    C. Preparation of growth factors and hepes buffer------------------------------ 25
        1. Retinoblastoma derived growth factor-------------------------------- 25
        2. Hepes buffer solution--------------- 26
        3. Nerve growth factor----------------- 26
    D. Method of creating wounds--------------- 27
        1. Retina------------------------------- 27
        2. Lens--------------------------------- 31
    E. Enucleations----------------------------- 33
    F. Fixation and embedding------------------ 34
    G. Sectioning------------------------------- 35
    H. Preparation of tissues for examination of lens lesions------------- 36

III. Results--------------------------------------- 37
    A. Daily clinical observations------------- 37
    B. Gross evaluations under dissecting microscope at time of enucleation-------- 42
    C. Gross observations of lens lesions------- 43
    D. Histopathologic observations of retinal lesions-------------------------- 47

IV. Discussion------------------------------------ 66

V. Conclusions----------------------------------- 71

VI. References------------------------------------ 73

Introduction

Recently, investigators from the Department of Ophthalmology and Visual Science at Yale University isolated and identified a growth factor from a human retinoblastoma cell line (Y-79)(Rubin et al., 1980). This factor was shown to promote growth in murine 3T3 cells and to stimulate DNA synthesis and mitosis in Nakano epithelial lens cells *in vitro* (Tarsio et al., 1980).

Nerve growth factor was isolated and characterized by Levi-Montalcini and colleagues in 1953 and it has been shown to promote growth on embryonic sensory nerve cells as well as on fully differentiated sensory ganglia (Levi-Montalcini, 1965).

Gospodarwicz and Moran, in a review of growth factors in 1976, stated that the test that allows one to conclude definitely that any growth factor is truly a mitogen of physiologic importance is demonstration that the growth factor is active *in vivo*.

The mitogenic effect of NGF is restricted *in vivo* to the early part of embryonic life (Levi-Montalcini et al., 1960). In the chick, hyperplasia of the sympathetic ganglia is best seen between days 7 and 10. Later, only hypertrophy is observed. In the mice, the mitogenic effect

of NGF is observed between days 3 and 9 post partum; after this, only hypertrophy is observed. Retinoblastoma derived growth factor has not been tested in an *in vivo* preparation. Neither NGF nor RDGF has been tested as to its effects in growth and differentiation in the eye. In fact, there is only one report in the current literature on the effects of a growth factor on the eye. Mesodermal growth factor from mouse submaxillary glands has been shown to have stimulating effects on the healing of corneal wounds in rabbits (Rich *et al*., 1979).

This project attempts to test the effects of NGF and RDGF in the healing of experimental wounds in the lens and retina of young pigmented Dutch-belted rabbits.

I. Review of the Literature

A. Wounds in the retina

Direct mechanical trauma is the obvious and probably the simplest way of creating wounds in almost any accessible tissue in the body. Trauma results in the physical interruption of blood vessels leading to hemorrhage, it causes cell destruction, and provides an avenue for foreign substances to gain access to the wound.

Immediately following the traumatic incident, wound healing begins to take place. Inflammation sets in, beginning with a vascular phase in which there is first vaso-constriction followed by vaso-dilatation and the release of the chemical effectors of inflammation. A cellular phase follows in which there is movement from vessels into the wound area of inflammatory cells. Inflammation is followed by repair, when the reparative cells of the tissue migrate and proliferate to cover the areas of wound created by trauma. Four types of cells are present in every tissue and these will respond differently in wound healing. There are: (1) progenitor cells that are involved in mitotic cycles, (2) immature cells that have ceased or are ceasing to divide and are preparing for tissue functions, (3) mature cells that are

always functional but cannot revert to mitosis and are approaching death, and (4) mature cells that may or may not be functional and that if circumstances dictate, are capable of reversion into mitosis. In almost every instance, epithelium responds to trauma by complete regeneration of cells and replacement of loss tissue. Nerve cells, however, do not undergo regeneration if the cell body is lost. Wound contraction is the last in the continuing steps of wound healing (Peacock, 1976).

The general pathology of human retinal inflammation is characterized by the vascular response (Hogan _et_ _al_., 1962; Yanoff _et_ _al_., 1975). The vessels in the inner layers of the retina become dilated, and leukocytes marginate and migrate into the tissues and perivascular spaces. Protein rich fluid accumulates in the perivascular tissues and edema results. The pressure exerted by the accumulation of fluid and cells soon leads to degeneration of retinal elements, forming large areas containing fibrin, cellular debris, inflammatory cells and lipoids. Macrophages from the tissues and blood accumulate in the cavities and become filled with debris and lipoid from the degenerating retinal cells. These soon depart from this location via the bloodstream or disintegrate in situ. Proliferation of connective tissue occurs from the region of the blood vessels and glia from the inner retinal layers producing a fibroglial scar. If red blood cells escape into the wound, the tissue will

stain positive for hemosiderin, especially after these cells have been phagocytized by macrophages. The retinal pigment epithelium (RPE) shows proliferation with and without metaplastic features in response to injury. The RPE may then die and its pigment phagocytized by macrophages.

In the rabbit, the vascular response in inflammation comes mainly from the choroid, since the inner layer of the retina is said to be avascular (Prince, 1964). Several investigators have created wounds in the rabbit (Rentsch, 1973; Foos, 1974; Foos & Gloor, 1975; Cleary & Ryan, 1979; Topping et al., 1979), rhesus monkey (Wallow & Tso, 1973), and owl monkey (Machemer & Norton, 1968; Machemer, 1978), or have studied the histopathology of traumatic wounds in human eyes (Smith, 1960; Eagling, 1975; Barry, 1975; Van Horn _et_ _al_., 1977; Machemer _et_ _al_., 1978). Because of the variables among clinical injuries, it has been difficult to generate specific information on the course of repair and proliferation in injured eyes, especially in the pathways proliferations tend to follow. An approach to the study of the response of retinal elements to wounds must consider the species, depth of the wound, method of creating the wound, the time elapsed, and the methods for studying the cellular response. In the following papers that I will discuss, I will specifically point out their findings in an attempt to develop a framework on which to later base our own observations on the effects

of RDGF and NGF on experimental wounds in the retina and lens of pigmented rabbits.

The first group of studies deal specifically with wounds that were created in an attempt to study the pathogenesis of wounds in the retina and the origin of preretinal and epiretinal membranes. Most of these experiments in which the reaction to wounds in the retina have been studied have focused specifically on the question of the nature of the epiretinal membrane formed. This has been so because of the clinical importance attributed to the formation of these membranes in the pathogenesis of retinal detachment.

Smith (1960) summarized what other authors had previously claimed to be the main cellular elements in the healing response of the retina. The list includes endothelial cells, pigmented or unpigmented cells of the ciliary epithelium, RPE, mesodermal elements of the retinal vessels, vitreous cells or from retinal glial cells.

Rentsch (1973) produced two full thickness retinal tears in the rabbit by destroying and removing the vitreous with a high speed rotating instrument and injecting silicone into the vitreous cavity. Pre- and retrobulbar bleeding recurred during the procedure. The animal was sacrificed 17 months later. A preretinal membrane was formed and studied with electron microscopy. This membrane consisted of extensions of Müller cells of astrocytic cells. The pathogenesis proposed is that once the hole in the

inner limiting membrane is created, Müller cells migrate into the epiretinal region through the hole. The astroglia then migrate and form attachments to the Müller cells.

Foos (1973) studied eight human eyes enucleated for orbital neoplasms. He found epiretinal membranes in eight eyes, and five eyes had breaks in the inner retina smaller than 50μ. The time at which these membranes began to form is unknown. These membranes, when studied under electron microscopy, were found to be exclusively formed of glial cells. Smith (1960) reported that epiretinal membranes are a common incidental finding in the microscopic examination of enucleated human eyes. He observed with light microscopic studies that the cellular constituents appeared to be fibrocytes or glial cells.

Foos (1975) made superficial lesions in the nonvascularized retina of six pigmented rabbits using a sharp microsurgical instrument. The eyes were enucleated at 1, 3 days and 1,2,6, and 10 weeks. They discarded any wound if the lesion went any deeper than the outer plexiform layer. Under electron microscopy they identified two processes of cellular reaction. As early as day 3 and up to day 14, a reaction they termed plexiform gliosis predominated. This consisted of side branches of Müller cells proliferating to form a layer about the perimeter of the wound. Also starting at day 3 but continuing and becoming more intense in the older wounds a process termed accessory gliocytosis took place. This consisted of

accessory glia adjacent to the wound becoming phagocytic and proliferating; following this their progeny migrated and progressively differentiated into fibrous astrocytes to fill in the wound. No vitreous was found in the wound. The disrupted inner limiting membrane failed to regenerate in any of the wounds.

Aaberg and colleagues (1970) observed that epiretinal membranes which were naturally occuring and experimentally induced in the owl monkey had varying cellular morphologic characteristics by light microscopy, resembling RPE in areas adjacent to retinal tears. Other investigators added that once in the vitreous cavity, the RPE cells can undergo rapid proliferation and metaplasia, forming aggregations of pigmented macrophages, fibrocyte like cells and frank epithelial cells (Machemer _et_ _al_., 1975; Mandelcorn _et_ _al_., 1975; Mueller-Jensen, 1975; Frayer, 1966).

Van Horn (1977) analyzed the cellular components of epiretinal membrane specimens from human eyes obtained by vitrectomy in rhegmatogenous retinal detachments. They found the membranes to be composed of glial cells and RPE like cells. Machemer (1978) in a similar study of human vitrectomy specimens found vitreous membranes to be composed of RPE which could look like fibrocytes, macrophages, or normal RPE and were observed to produce collagen strands.

Machemer and colleagues (1968), in an attempt to

produce experimental lasting retinal detachment, studied wounds created by means of injecting a jet stream of vitreous as close to the retina of owl monkeys as possible, but without touching it. The enucleated the eyes at one hour, 1 and 3 days, and 1,2,4,8,12, and 20 weeks. They found that in the retinal hole, the edges rolled inward secondary to the retinal edema. By day one, the inner segments and outer segments of the photoreceptors were destroyed by the mechanical injury and the nuclei appeared pyknotic. By day 3, the RPE that had been destroyed was covered by flattening of the remaining RPE around the wound and proliferation of the RPE, which in some wounds was so excessive as to be several cell layers deep. In the peripheral retina, by day one, there was loosening of the inner layers with much edema fluid. The whole retina was seen thicker, with horizontal structures such as the nerve fiber layer and portions of the inner plexiform layer being less edematous. Changes were noted which progressed from day one through day 14. The inner nuclear layers showed vacuolation and cystoid formation. The retina became more ondulated probably secondary to the increasing edema. The neuroepithelium became irregular with more degeneration in the outer segments than in the inner segments. Macrophages were abundant in this region. Starting at 7 days and certainly by 14 days, the outer nuclear layer showed signs of degeneration, particularly in areas of frank separation

between the neuroepithelium and the RPE.

Cleary and Ryan (1979) injured the rabbit fundus through an incision in the pars plana and a transvitreal approach. They observed wound healing beginning at 3 days and was well established by 9 to 12 days. Vitreous was seen incarcerated in the wound. The areas with detached retina showed degenerative changes with loss of photoreceptors and intraretinal cystoid swelling. In some peripheral areas, the retina was markedly ondulated. In detached retinas, the RPE was seen to proliferate as early as 15 days. It showed diffuse hyperplasia with sheets of flattened pigmented cells and plaques of partially depigmented spindle shaped cells. By 21 days there was marked proliferation in the outer retina of glial cells. These cells were also seen to displace the outer retinal layers from the external limiting membranes. It is important to note that the RPE was not observed to proliferate in retinas that were not detached. They observed that in rabbits eyes, epiretinal membranes were not common and were probably glial in origin.

Topping and colleagues (1979) injured the rabbit retina by inserting a probe through the sclera and retina into the vitreous, one disc diameter inferior to the optic disc. They sacrificed the rabbits 30 minutes, 1,4,7,14,30, and 60 days after the injury. They observed the healing process in the sclera and retinal wounds with ligh microscopic sections. They found an acute inflammatory

reaction starting a few hours and up to 4 days after the lesions had been made. This reaction was composed of neutrophils, proteinaceous exudate and macrophages in the wound area and by day one, the choroidal fibrocytes were seen to change morphologically as if getting ready to start proliferation. By day 4, there was an extensive reparative process, with much fibroblastic proliferation in the wound area. Metaplastic RPE had migrated to form a sealing scar between the torn retinal edges. The inner and outer nuclear layers showed much degeneration and loss in a progressive manner starting at day 4.

Other investigators (Eagling, 1975; Barry, 1975) have pointed out the importance of bleeding into the wound and vitreous, and vitreous incarceration in the evolution of the healing of retinal wounds. With vitreous hemorrhage, there is pigment cell deposition and thickening of the posterior vitreous face, causing traction on the retina leading to detachment. With vitreous incarceration in the wound, the vitreous becomes adherent to inner aspects of the wound, with secondary fibrosis of the vitreous from the wound.

Some investigators have studied the wounds in the retina created by xenon arc photocoagulation (Curtin _et al_., 1963; Wallow _et al_., 1973) and by argon laser photocoagulation (Apple _et al_., 1973). In photocoagulation of the human retina, an attempt is made to create a lesion involving the RPE, photoreceptors, outer nuclear layer, and outer

plexiform layer in order to induce the formation of a chorio-retinal adhesion. Bruch's membrane is left intact, and no direct contribution to wound healing is made by the choroid. The RPE is observed to proliferate becoming metaplastic, phagocytising the remnants of the degenerating photoreceptors. In lesions in which the full thickness of the retina was burned, hyperplastic glial cells originating from the viable edges of the wound formed a glial scar. In lesions involving the outer half of the retina, hypertrophic Müller cells located internal to the necrotic photoreceptor cells formed cytoplasmic extensions that slowly filled the defect of the outer nuclear layer (Wallow _et_ _al_., 1973; Curtin _et_ _al_., 1963; Wallow _et_ _al_., 1975).

B. Wounds in the lens

Numerous investigators have looked at the influences the neural retina exerts over the development, maturation and maintenance of the structures of the lens (Coulombre, 1965). Lens inversion experiments have shown that the epithelium, lens cells and fibers will alter their growth and development depending on which side, the vitreous or aqueous, they are facing (Coulombre, 1963; Yamamoto, 1976). More direct experimentation on the role of the retina on lens cell differentiation definitely point to some factor secreted by the retina influencing the lens epithelial cell growth (Muthukkaruppan, 1965;

Arruti *et al*., 1978). Other investigators have shown that fetal eye tissue when implanted into the evacuated lens region in the mature eye will develop into a normal lens (Chanterrishvili *et al*., 1961; Pettit, 1963).

Most recently, *in vitro* experiments have shown the potential of a retinoblastoma derived growth factor to stimulate DNA synthesis and mitosis in Nakano epithelial lens cells (Tarsio *et al*., 1980). It has been observed in humans that when the lens capsule is lacerated, the wound borders gape and have a tendency to roll outward. Following discission, the subcapsular epithelium may proliferate to produce abortive lens fibers. When damage is done to the anterior subcapsular epithelium and deeper lens cells, they take the shape of large globules called Elching's pearls. Clinically, they appear as a cluster of fish eggs. When damage is done to the posterior subcapsular region, there is thickening of the posterior capsule and proliferation of lens fibers and fibroblasts (Hogan & Zimmerman, 1962). These changes following accidental injuries to the lens will lead to the development of a cataract (Davidson, 1940).

Harding (1961) observed that under abnormal conditions the mitotic activity of the lens epithelium may be increased. After trauma to the subcapsular epithelium, the first cells to begin mitosis are those adjacent to the wound, but the area is seen to enlarge gradually for 28 to 32 hours, the extent of the spreading activity being related to the size of the wound.

For the study of the effects of RDGF on experimental wounds *in vivo*, we used the Dutch pigmented rabbit model with posterior discission of the lens capsule and fibers. The rabbit eye is smaller and more spherical than the human eye, and the lens is large in relation to the total size of the eye and extends almost 3mm posterior to the limbus (Prince, 1964). The capsule, subcapsular epithelium and lens fibers are distributed in the same manner as in humans.

When comparing findings in animal experiments to humans, Rafferty (1975) noted that the animal lens reacts somewhat differently to injury. Discission of the rabbit lens does not result in early and complete opacification. In the rabbit, the wound in the posterior capsule does not gape as it does in man and thus, wound healing is facilitated (Vogt, 1931). The factors or mechanisms involved in this rapid healing are not well understood.

Fisher and colleagues (1976) performed a posterior capsulotomy on the rabbit lens. Slit lamp examination at 8 months showed that the hole in the capsule remained patent and that new growing fibers beneath the capsule either cross the exposed surface or stop at the margins of the hole. Few opacifications were seen. Others have also reported the difficulty encountered in creating traumatic cataracts in the rabbit lens (Fagerholm *et al.*, 1979).

When large enough lesions are created in the capsule, protrusion of cortical lens fibers, opacification and cataract formation may be seen. Rupture of the anterior subcapsular epithelium will lead to leakage of sodium and water through the local epithelial defect and in between the subcapsular lens fibers, leading to hydration and swelling of the fibers (Riley, 1970). Unless the epithelium can grow back fast enough to seal the defect, a permanent cataract will be formed.

We decided to test RDGF in the healing of experimental wounds in the rabbit lens because of the speculative role of RDGF as a factor which is normally released by the retina for the continued growth of epithelial cells of the mature lens.

C. Growth factors

Two recent literature reviews on growth factors (Nevo *et al.*, 1979, Gospodarowicz *et al.*, 1976) have attempted to formulate a definition of what a growth factor is. In the first, a growth factor is defined as a growth stimulating substance that is not an essential nutrient. In the second, it is defined as a substance that acts to increase cell size and rate of proliferation as well as matrix production and to prolong cell survival.

A list of the growth promoting substances in the recent literature shows that the number is ever increasing. Aside from the growth promoting substances that have

been known for a long time such as growth hormone, thyroid hormone and other substances from the gonads and pancreas, the newer list would include: somatomedians, A,B,C, nonsuppressible insulin-like activity factor (NSILA), multiplication-stimulating activity factor (MSA), nerve growth factor (NGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), overgrowth stimulating factor (OSF), liver growth factor (LGF), ovarian growth factor (OGF), cartilage growth factor (CGF), and chalones (Nevo *et al*., 1979). The newest addition to the list is retinoblastoma derived growth factor (RDGF)(Rubin *et al*., 1980).

All these growth factors have been isolated from human or animal tissues and their activity demonstrated in *in-vitro* cell culture preparations by their ability to induce growth and proliferation in the absence of other identifiable growth promoting substances. The definitive test that allows one to conclude that any growth factor is truly a mitogen of physiological significance is to demonstrate that the growth factor is active *in vivo* (Gospodarowicz *et al*., 1976). For our study, we chose the two growth promoting substances that we think might have some specificity for neural and specifically ocular tissue: NGF and RDGF.

1. Nerve growth factor (NGF)

In 1951, Levi-Montalcini & Hamburger reported that a soluble factor released from sarcoma

tumor cells elicited a growth promoting effect on the sympathetic ganglia of chick embryos. They called this substance a nerve growth factor (NGF). In 1960, Cohen was able to purify a NGF from mouse salivary glands which was studied biochemically and found to be more potent than the previously isolated NGF from the sarcoma tumor. Since then, other tissues have been found to contain this NGF (Banks _et al_., 1973).

NGF from the mouse salivary gland has been characterized as a dimer of two identical peptides each consisting of 118 amino acids with a molecular weight of 13,260 daltons and containing three disulfide bonds (Hogue-Angeletti _et al_., 1975; Young _et al_., 1976).

I.A. Hendry and colleagues (1974) noted that NGF does not increase the number of neurons in the sympathetic ganglia by enhancing their multiplication, but they point out that the marked increase in the number of neurons is due to the survival of redundant immature neurons that would ordinarily die off in the course of development. The effect of experimentally administered NGF is to enable the redundant neurons to survive and differentiate in spite of their failure to establish connections. Sensory nerve cells of certain ganglions are receptive to NGF only during a restricted period of their development, later becoming refractory (Levi-Montalcini _et al_., 1979). It has been shown that the existence of specific receptors on the neuronal surface makes it possible for NGF to exert

its effects at exceedingly low concentrations of about 2.8 micrograms per liter (Banerju *et al.*, 1973).

Another functional characteristic of NGF that has been observed recently is that certain non-neuronal cells when exposed to NGF respond by acquiring properties characteristic of sympathetic neurons (Greene & Tischler, 1976). Increased blood levels of NGF have been found in humans with neurofibromatosis, medullary carcinoma of the thyroid, Paget's disease of bone and familial dysautonomia (Nevo *et al.*, 1979).

To our knowledge, no work has been published to test the response of either *in vivo* or *in vitro* retinal tissue to NGF.

2. Retinoblastoma derived growth factor (RDGF)

RDGF was isolated from medium conditioned by serum-free growth of a human retinoblastoma cell line, Y-79, after they had been growing a serum-free environment for over five months (Rubin *et al.*, 1980). The Y-79 cells had been obtained from a human retinoblastoma tumor of a 2-1/2 year-old girl in 1971 (Reid *et al.*, 1974).

This growth factor has been partially characterized by Sephadex 100 chromatography as a protein with an approximate molecular weight of 38,000 daltons. The substance is heat labile at a temperature of 60°C for 10 minutes. It shows a slight decrease in its activity when kept at 37°C for periods longer than twelve hours.

Trypsin digestion has been shown to inactivate the factor (Rubin *et al.*, 1980).

RDGF was shown to stimulate both cell division and thymidine uptake in Swiss mouse 3T3 fibroblast. Maximal incorporation of ^{3}H-thymidine is obtained between 16 and 40 hours. The investigators who isolated and characterized this growth factor noted than when the medium in which the cells producing this growth factor is changed too frequently or in too high a percentage, the Y-79 cells die. They assume that this results because of the removal of the growth factor that the Y-79 cells are producing for their own growth and maintenance.

RDGF has been tested for its growth promoting characteristics in several *in vitro* systems. It appears to stimulate growth changes of retinal cells in culture. Tarsio and colleagues (1980) showed that this factor has growth promoting activity for Nakano mouse lens epithelial cells. They obtained stimulations of up to 9-fold with less than 0.2μg/ml of partially purified factor which was equivalent in activity to 2% fetal bovine serum. Lack of sufficient RDGF may be an important reason why other retinoblastoma cell lines have not been successfully grown in tissue culture.

RDGF has not been tested in any *in vivo* models. Other than its growth promoting activity of Y-79 cells, Nakano mouse lens epithelial cells, and the preliminary

result of Rubin and colleagues which show that the growth factor will support the growth of normal retina explants, RDGF has not been tested in an *in-vivo* animal retinal preparation.

II. Materials and Methods

A. Experimental animal

The Dutch pigmented rabbit was chosen as the *in vivo* model for many of the same reasons that other investigators have used this animal (Abrams *et al.*, 1978; Prince, 1964; Topping *et al.*, 1979; Rentsch, 1973; Algvere, 1976; Rich *et al.*, 1979; Cleary *et al.*, 1979). The rabbit eye is smaller than the human eye and is nearly spherical. There is essentially no pars plana and the ora serrata is only 3mm posterior to the limbus. The lens is large in relation to the total size of the eye and extends almost 3mm posterior to the plane of the ora serrata. The fundus of the Dutch pigmented rabbit is pigmented whereas that of the albino is off white and, therefore, anatomical landmarks are more difficult to identify.

When one looks at the Dutch pigmented rabbit fundus, one sees that the optic nerve is above the posterior pole of the globe, is deeply cupped and horizontally oval in shape. Extending from it nasally and temporally are the medullary rays and these extend up to the region of the equator. The arterial pattern exiting from the optic disc is confined primarily to the area of the medullated fibers. No vessels are seen to extend beyond this white background, and the remainder of the retina is said to be avascular (Prince, 1964).

Below the disc is a horizontally elongated area clear of vessels which is used for central and most acute vision. This corresponds to what is called the "visual streak" and to some extent, it possesses macular qualities (Prince, 1964).

Young (15-20 week), male, pigmented Dutch-belted rabbits weighing approximately 1.0 ± 0.25 kg were used. As mentioned before, the pigmented, in comparison to the albino rabbit, offers a better visualization of the fundus. Young animals were chosen under the assumption they may have more repair and regenerative capacities (Jacobson, 1971) and they may be less likely to have age-related changes in their retinas (Lai, 1978).

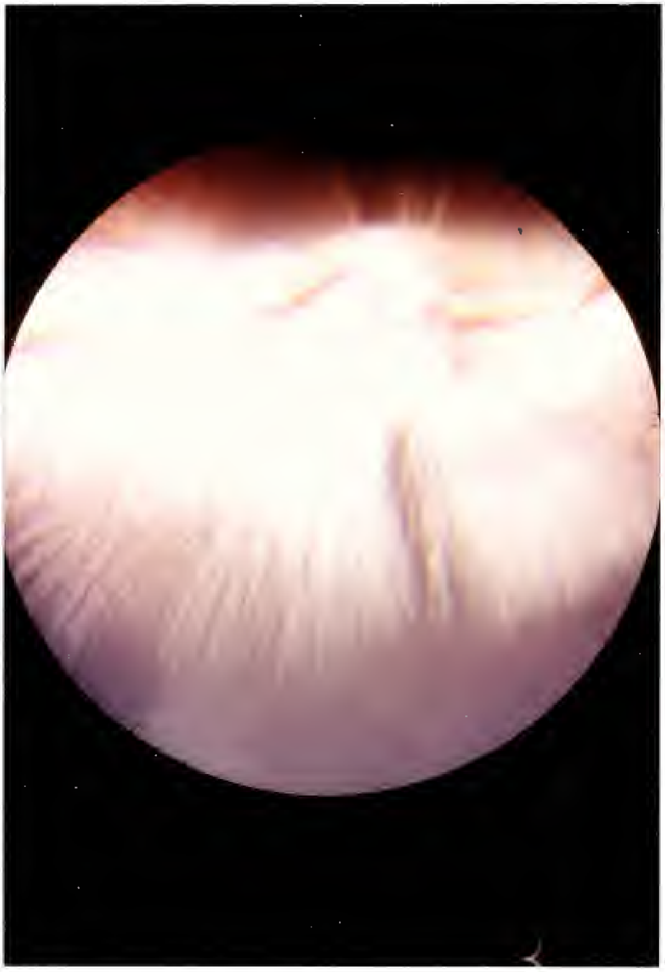


Fig. 1 Normal Dutch Pigmented Rabbit Fundus. Photograph shows area of myelinated nerve fibers. The arterial pattern exiting from the optic disc is primarily confined to this area. No vessels are seen to extend beyond this white background and the remainder of the retina is avascular.

B. Anesthesia

One drop of phenylephrine hydrochloride 10% was instilled in each eye followed by one drop of cyclopentolate hydrochloride 1%. This combination achieved fast and sustained mydriasis for the length of time required for the operative procedure.

Next, two drops of proparacaine hydrochloride 0.5% were instilled in each eye as a topical anesthetic. Following this, an intramuscular injection of ketamine hydrochloride, 9mg/kg, was injected slowly into the hind thigh musculature. Approximately 15 minutes after injection, the animals became unresponsive to pain stimulation. They were transferred to the operating table and wrapped snuggly with a towel to decrease the possibility of involuntary movement. A retrobulbar injection of 1 ml of lidocaine hydrochloride 1% was injected using a long 21g. needle. At this point, the fundus was examined with an indirect ophthalmoscope to check for abnormalities or inadvertent trauma to the fundus. This anesthetic protocol was followed with each rabbit and was found to be adequate to maintain the animal comfortably anesthetized for the duration of the procedure. Only twice did we find it necessary to give an additional dose of ketamine.

C. Preparation of growth factors and hepes buffer.

1. Retinoblastoma derived growth factor

RDGF was recently identified and characterized, and its activity assessed *in vitro* (Rubin *et al*., 1980; Tarsio *et al*., 1980). The Y-79 human retinoblastoma cell line from which it was isolated has been in continuous cell culture since being established from the eye tumor of a 2-1/2 year-old white girl in 1971 (Reid *et al*., 1974). The Y-79 cell cultures were maintained in RPMI-1640 supplemented with 5% fetal bovine serum, 3nM Na_2SeO_2 and 100μg/ml Kanamycin sulfate in Corning 490cm^2 roller flasks in a total volume of 50ml at 37°C in a moist atmosphere of 95% air and 5% CO_2. The roller flasks were not rotated. Cultures were fed twice weekly and split 1:2 every three weeks. During feeding, approximately 10ml of the depleted medium was left in the flask.

The cells were adapted to a completely defined medium by gradually weaning them off of their dependence for fetal bovine serum (FBS) according to the following schedule: two weeks each in medium supplemented with 3%, 2% and 1% FBS and three to four weeks in 0.5% prior to transfer to serum-free medium. The serum concentration was reduced only in those flasks in which the cells appeared healthy. The serum-free medium also contained 0.01M Hepes (N-2-hydroxyethylpiperazine-N-2, ethanesulfonic acid).

Twice weekly, 35ml of Y-79 conditioned serum-free medium was removed per roller flask. Cells and subcellular

particles were removed by centrifugation for one hour at 70,000 x g at 0°-5°C. The supernatant was concentrated 100X in an Amicon Model 52 concentrator using a PM-10 filter. The concentrate was stored at -15°C. Immediately prior to use, it was diluted to 70X and 7.1X concentrations with Hepes-saline buffer (0.01M Hepes, 0.15M NaCl, pH 7.0) and filter sterilized with a 0.22μ Millipore filter. These two aliquots of RDGF were stored at -15°C until ready to use in the rabbit eyes.

2. Hepes buffer solution

Hepes-saline buffer was made by using a concentration of 0.01M Hepes (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid), 0.15M NaCl at pH 7.0; all diluted in approximately 40cc of 1X distilled water that was passed through the Millipore Milli-Q ion-exchange column and further purified by filtering it through a 0.22μ Millipore filter followed by glass distillation. Approximately 40μl of Hepes-saline buffer were stored at -15°C until ready to be used in the rabbit eyes.

3. Nerve growth factor

NGF (7S) was purchased from Collaborative Research and was dissolved at 10μg/ml in RPMI-1640 containing 0.1% fetal bovine serum and filter sterilized with 0.22μ filter. Approximately 40μl of NGF was stored in a glass flask at -15°C until ready to be used in the rabbit eyes.

Before these solutions were injected into the rabbit eyes, they were removed from the -15°C freezer and thawed to 37°C in a warm waterbath.

D. Method of creating wounds

1. Retina

The rabbit was anesthetized, placed on the operating table, and the eye was proptosed using a scleral depressor against the lower eyelid. Proptosing was maintained by securing the eye in a hole made in a latex surgical glove. The superior rectus muscle was identified and securely held with a tissue forcep. A 6.0 nylon suture with an atraumatic needle was passed through the whole width of the muscle. The suture was cut at a length of approximately 4 inches and the ends held together with a Mosquito forcep. A suture was placed in the samme manner through the lateral rectus muscle. With these two sutures in place, the eye could be moved in all directions and also securely immobilized when necessary.

A hole through the conjunctiva and sclera was made approximately 2mm posterior to the limbus and nasally between the superior and medial rectus muscles, with a 23g needle. A minimal amount of vitreous evulsed from the hole.

The Machemer vitrectomy instrument was primed with a running drip of Ringer's lactated solution. It was introduced through the hole made and the tip immediately

visualized with indirect ophthalmoscopy. The instrument was angled at 45° towards the posterior pole. Care was taken not to traumatize the lens but this was not always possible. The eye was moved until the optic disc and the medullated nerve fibers were clearly seen. The cornea was kept moist with BSS solution.

The needle of the vitrectomy instrument was slowly and cautiously advanced towards the posterior pole under constant visualization with the indirect ophthalmoscope. Three to four small lesions were made below the medullated fibers nasally, in the area which corresponds to the visual streak.

The vitrectomy instrument was then removed and a 10μl Hamilton microsyringe was introduced through the same hole in the conjunctiva and sclera and the tip of the needle moved as close as possible to the posterior retina. Approximately 10μl of either RDGF 71X, RDGF 7.1X, NGF, or Hepes was injected. The syringe was removed and the eye returned to its socket after removal of the extra-ocular muscle sutures. The scleral wounds and the conjunctiva were not sutured. Neither sterile instruments nor post-operative antibiotics were used (Machemer _et_ _al_., 1968). All the animals were returned to their cages in satisfactory condition.

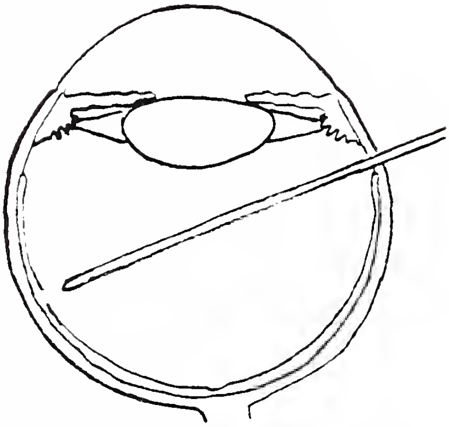


Fig. 2 Surgical procedure for lesioning the retina. The Machemer vitrectomy instrument is introduced through the pars plana and advanced in the vitreous cavity until the tip injures the retina.

Previous to developing the above technique for creating the experimental wounds in the retina, we had tried two different methods. First, we used an instrument which consisted of a 30g piece of wire introduced through a 23g needle under direct ophthalmoscopy. When these wounds were evaluated histologically, it was observed that the depth of the wound was quite variable from eye to eye. This was attributed to the lack of binocular vision afforded by the direct ophthalmoscope. The second method which we tried was using the same 30g wire through the 23g needle but using a four mirror contact lens and the Zeiss operating microscope. Problems were encountered in keeping the contact lens in its proper position because of its size and the area of sclera it covered. This made it difficult for us to penetrate into the vitreous cavity without damaging the peripheral retina.

Schedule for Injection and Enucleation of the Rabbit Eyes

Day	HEPES	RDGF 71X	NGF	RDGF 7.1X
1	1 RE	1 LE	5 RE	5 LE
4	2 RE	2 LE	6 RE	6 LE
7	3 RE	3 LE	7 RE	7 LE
14	4 RE	4 LE	8 RE	8 LE

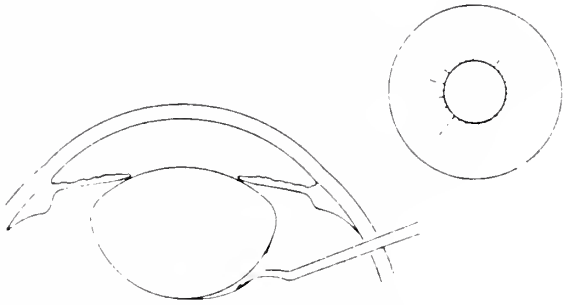
Total: 8 rabbits, 16 eyes

2. Lens

For this aspect of this research, the rabbits were anesthetized and prepared in the same manner as described above. The vitrectomy instrument was introduced through the conjuntival-scleral hole. The tip of the needle was smoothly run over the posterior pole of the crystalline lens making a linear pattern. The vitrectomy instrument was then removed and the Hamilton microsyringe was introduced and either Hepes or RDGF 71X was injected into the vitreous.

Lens Lesions

Day	HEPES	RDGF 71X
1	1 RE	1 LE
7	2 RE	2 LE
14	3 RE	3 LE



A— Surgical procedure

Fig. 3 Surgical procedure for injuring the posterior lens capsule. The cutting instrument is introduced through the pars plana and a slit is made in the posterior capsule.

E. Enucleations

The eyes were enucleated following the schedules presented above. Each rabbit was anesthetized using sodium pentobarbital through a peripheral ear vein. Using dissecting scissors each eye was enucleated avoiding damage to the globe. These were quickly placed in ice water and with cutting scissors were denuded of their adnexa, including most of the conjunctiva. This procedure appreciably reduced any post-mortem changes (Grierson et al., 1976). A small incision with a sharp blade was made approximately 2mm behind the limbus and the incision was carried circumferentially with the cutting scissor. Care was taken not to apply pressure to the globe and cause massive extrusion of the vitreous. The anterior part of the eye was carefully lifted and placed en bloc in the glutaraldehyde-formalin fixative solution. The posterior part of the eye was then placed in a petri dish containing fixative solution. Under the magnification of the dissecting microscope, the vitreous was removed by using a fine tipped cotton applicator, with special precautions as to not detach the retina. In a few of the specimens, fibrous bands and epiretinal membranes were cut with a sharp blade in order to avoid traction on the retina when removing the vitreous.

The fundi were scanned with the dissecting microscope until all lesions were identified. The fundi was then sectioned using a #10 scalpel blade. A drawing of the shape of the tissue and the lesion was made since all

gross detail would be lost when the tissue was placed in 1% OsO_4. The pieces of tissue containing the lesions were then placed in bottles containing cold glutaraldehyde formalin fixative and refrigerated. All eyes were enucleated following the same procedure. All animals were sacrificed after enucleating both eyes.

F. Fixation and embedding

Tissues were first fixed in a solution consisting of 2.5% glutaraldehyde, 2.0% formalin and 0.1M phosphate buffer at a pH of 7.4. This was kept in ice for approximately three hours. The tissues were then washed in a solution of 0.133 M phosphate buffer and 5% sucrose at pH 7.4. This was done twice for five minutes each time.

Subsequently, the tissues were post-fixed in two different solutions: one consisting of 1% OsO_4 in 0.1M phosphate buffer and 5% sucrose at pH 7.8 and the second consisting of 1% OsO_4 in 1.5% potassium ferrocyanide. They remained in this solution for approximately 120 minutes in a shaker bath.

Next came the dehydration step. The tissues were placed in 70%, 85%, 95% and 100% EtOH. They were placed twice in the 70%, 85%, and 95% EtOH for a period of five minutes each, and three times in fresh 100% EtOH for ten minutes.

The tissues were then cleansed in Toluene for one hour, making a change into new solution after the first

ten minutes.

Next, came the infiltration step using Spurr's medium (Spurr, 1969). This is a low viscosity embedding material which is made from the following commercially available materials:

CIBA RD-4 (VCD)	10g
DER 736	6g
NSA	26g
DMAE	.4g

In order to produce a softer or harder block, more or less DER 736 was mixed, and the color could be made lighter or darker by decreasing or increasing the DMAE.

To infiltrate the tissues, the specimen bottles were thoroughly cleansed of the Toluene with a pipette. The Spurr's medium was added to the tissues and left for three hours at room temperature.

Using the drawings previously made of the tissues and the lesions, the specimens were placed in warmed rubber molds in the correct orientation to ease and minimize the work of sectioning. The molds were filled with the Spurr's medium and numbered. They were then transferred to the oven were they were left at 60°C for approximately 18-24 hours. A test block was removed at this time and checked for hardness.

G. Sectioning

Once the molds were removed from the oven, they were allowed to cool at room temperature. The blocks were

then removed from the molds and placed in chucks where they were trimmed and the tissues oriented as close as possible to the cutting edge of the block.

An Ultratome III, LKB model 8800, microtome was used. Once the proper 1µ serial sections were obtained, these were placed on a glass slide. Methylene blue 1% and Azure II 1% in equal parts were added and left for approximately 20 seconds for staining. A cover slip was then placed over the slide. The section was then examined under the light microscope. Serial sections were obtained of most of the lesions.

H. Preparation of tissues for examination of lens lesions

The lenses with the lesions were placed in glutaraldehyde-formalin fixative solution and were divided into two groups, each containing both experimental and control lenses. One group of lenses were thin sectioned and stained with H + E. The second group of lenses were prepared for scanning electron microscopy.

III. Results

A. Daily clinical observations

All animals were examined post operatively. An indirect ophthalmoscope with a 30D lens was used. There was no evidence of retinal detachment other than in the areas surrounding the lesions and this was usually limited to no more than 1mm circumferentially around the lesion. There was slight pallor around the lesion. All lesions showed minimal bleeding into the wound cavity, which eventually was observed to disperse into the adjacent vitreous. There was no active bleeding observed. A track was seen to form in the area of the vitreous where the instrument and injection needle had been introduced. A few hours after the lesions had been made, some cloudiness of the vitreous was observed, probably corresponding to the same areas where the control solution and the growth factors were injected. No vitreous was observed to come out through the scleral hole.

Fundus pictures were taken of one of the rabbits to illustrate the location, magnitude, and clinical appearance of the wound.

Fundus drawings were made at one day post op in order to document and follow the gross appearance of the wounds.

Indirect ophthalmoscopy was performed every post op. day and results recorded. The fundus hemorrhages observed right after the injury cleared slowly and subretinal blood

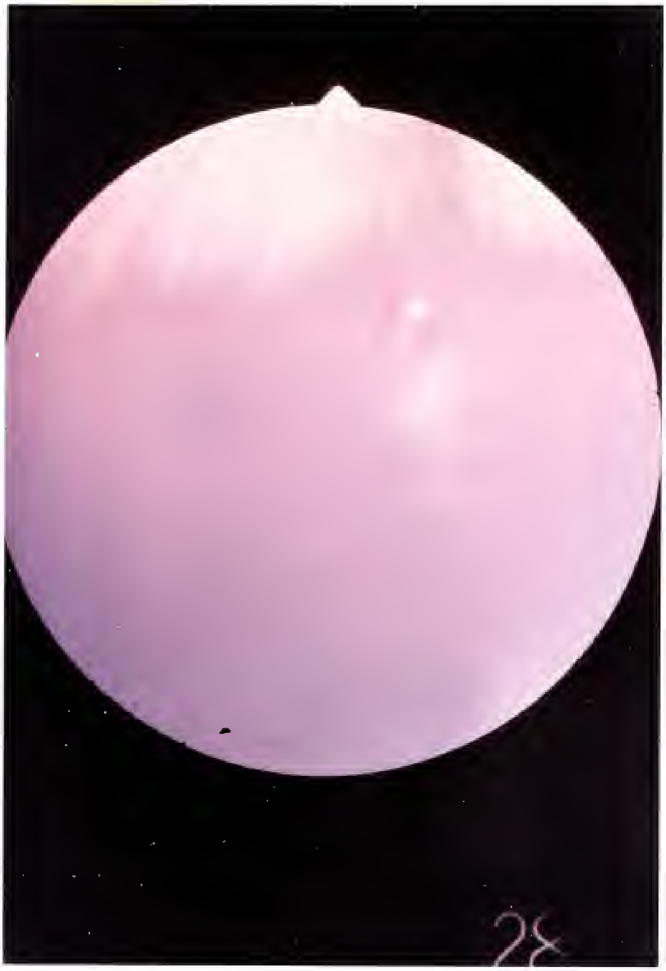


Fig. 4 Lesions in Rabbit Fundus 1. Lesion approximately one hour after injury. The lesion is one disc diameter below the optic disc, in an avascular area. Slight vitreous bleeding around the wound is present.

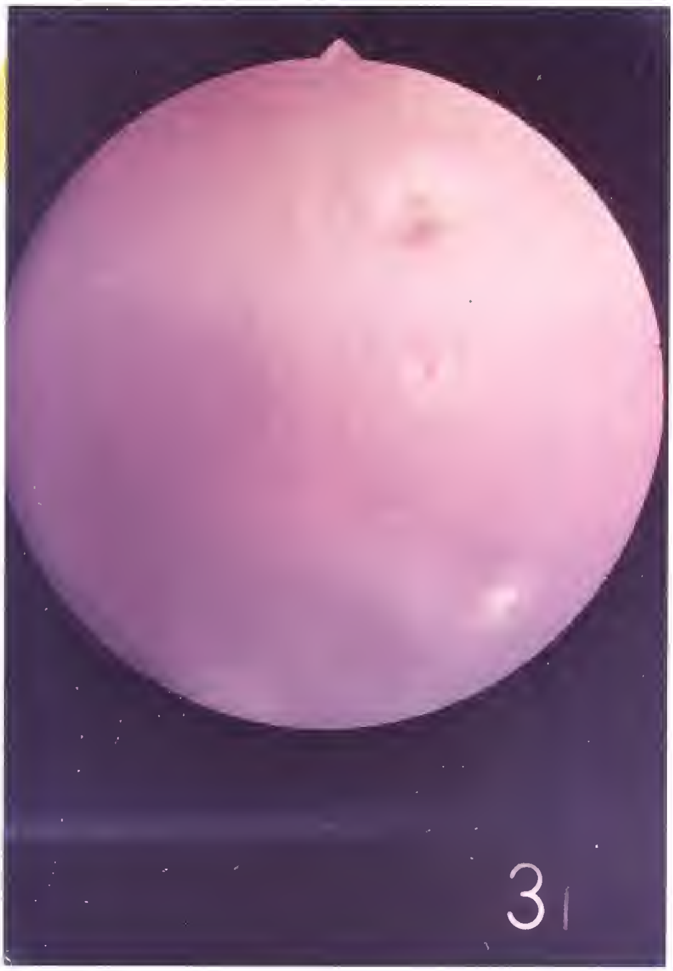
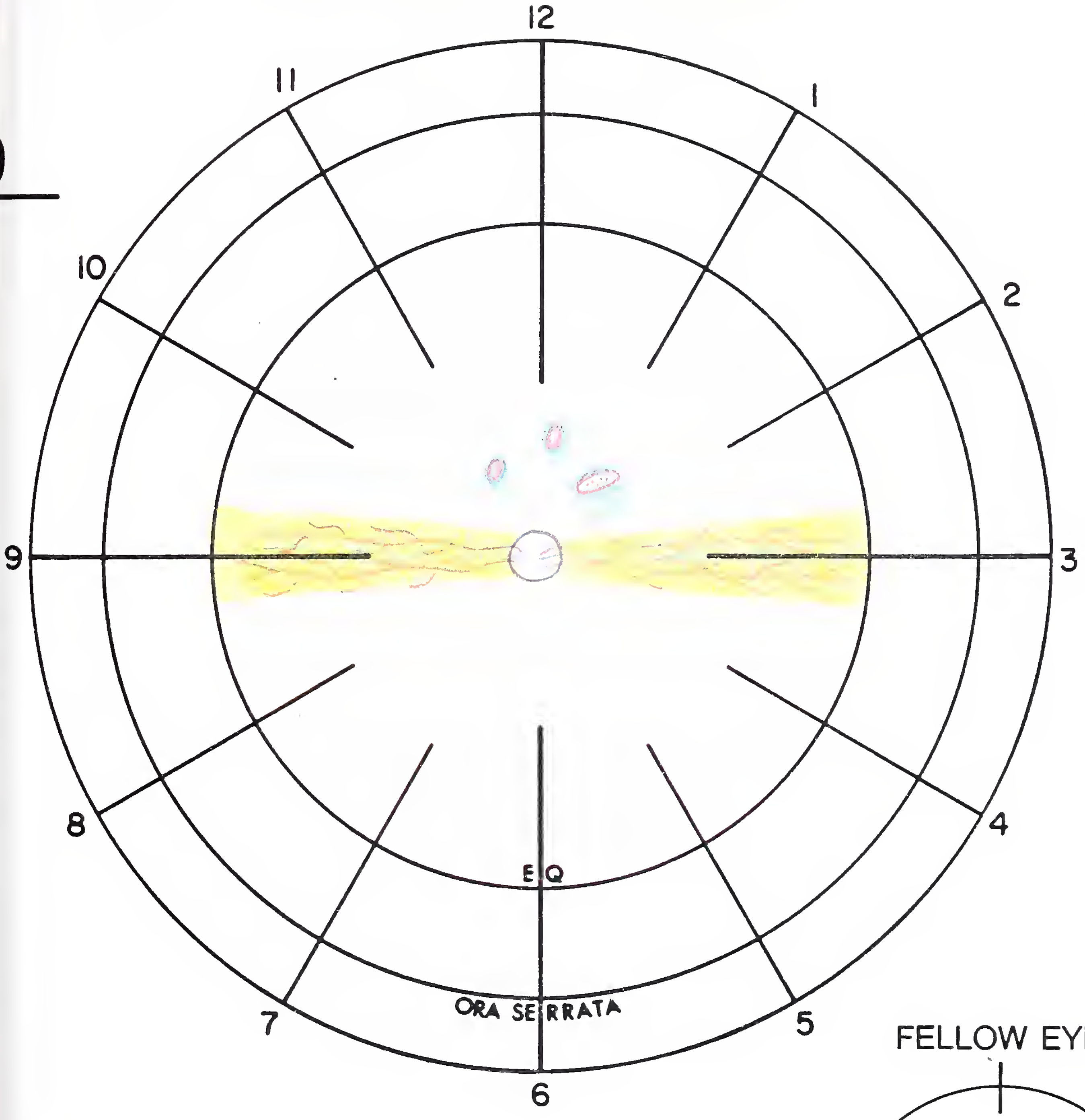
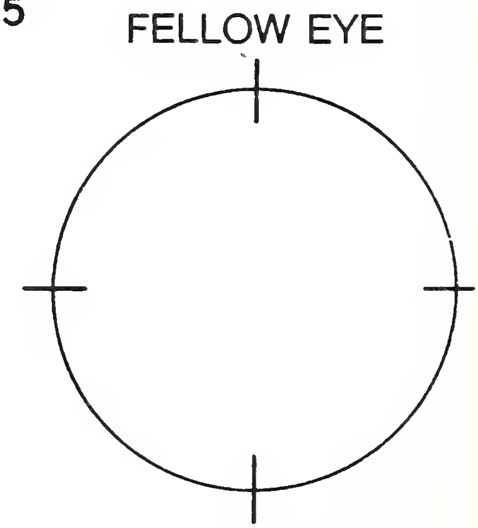


Fig. 5 Lesion in Rabbit Fundus II. Three lesions approximately one hour after injury. The location is more peripheral than in Fig. 4. There is some bleeding into the vitreous.

R. JOEL L. LEVIN
ETINA STUDY CENTER

YALE DEPARTMENT OF
OPHTHALMOLOGY





ate:
umber:
ame:

Fig. 6 Fundus drawings of retinal lesions. The following color and symbol code was used:

Red lines - normally attached retina
Blue lines - detached retina
Red vascular pattern - retinal arterioles
Blue vascular pattern - retinal veins
Red spot circled with blue - retinal tear
Red spot - retinal hemorrhage
Yellow - myelinated nerve fibers
Green - any opacity including blood in the vitreous
Black - melanin pigment

was occasionally visible even 14 days after injury.

In the lenses, a lesion was observed in the posterior capsule. Marked cloudiness and membrane formation were observed. These were first seen at day 7 post op and became more noticeable at day 14. In two of the eyes where lens lesions were made, the fundus could not be seen at day 14 due to the cloudiness of the posterior capsule.

The rabbits were examined daily. The degree of light perception was tested by means of pupillary response to light. There was no significant change in this parameter from day 1 to day 14 post op. There was no indication of external infections as evidenced by suppuration. Healing of the scleral wound was accomplished by a thin grayish white membrane that covered the entrance wound. The retina did not seem to detach substantially after injury, though some serous and serosanguinous elevation of the retina encircled the retinal wound immediately following the injury. By one week the retina was flat and much surrounding subretinal blood was absorbed. The retinas remained attached for the remainder of the experiment. At this indirect ophthalmoscopic level, no significant differences could be observed between the experimental and control eyes.

B. Gross evaluations under dissecting microscope at time of enucleation

In the eyes enucleated at day 1 there was no appreciable difference observed in the wounds between the

control and experimental eyes. Both showed a well demarcated blood clot in the center of the lesions, minimal retinal detachment with subretinal serosanguinous fluid, and areas of slight pallor directly adjacent to the lesions. The vitreous was clear, showing only some cloudiness close to the lesion, probably at the site where the solutions had been injected. There was no fibrous band formation.

At day 4, one eye with the injection of RDGF 7.1X showed dark dot-like markings in the periphery and the central area of one of the lesions. The rest of the retina looked slightly depigmented. The other lesions showed no change from the observations made for day one. The vitreous showed more cloudiness inferiorly, close to the area of the retinal wounds.

The retinal wounds from the eyes enucleated at day 7 and day 14 after the operation showed no striking difference other than progressive resorption of epiretinal blood and increasing cloudiness of the vitreous in the areas close to the wounds. Again, there was no marked difference between the experimental and control group.

C. Gross observations of lens lesions

The rabbit lenses two weeks after having made a lesion through the posterior capsule, showed some differences between the lenses which had been injured followed by an injection of the retinoblastoma derived growth factor and those injured followed by an injection of Hepes buffer as a control.

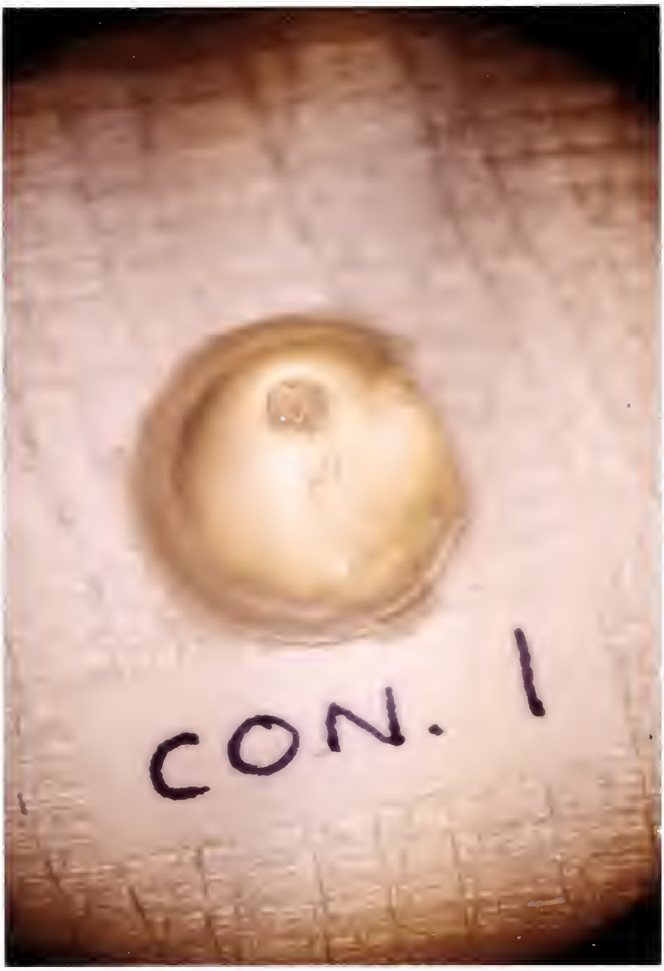


Fig. 7 Photograph showing control lens. Note slit-like wound extending vertically over posterior capsular region. Irregularities on the right equatorial area is due to handling of the specimen.

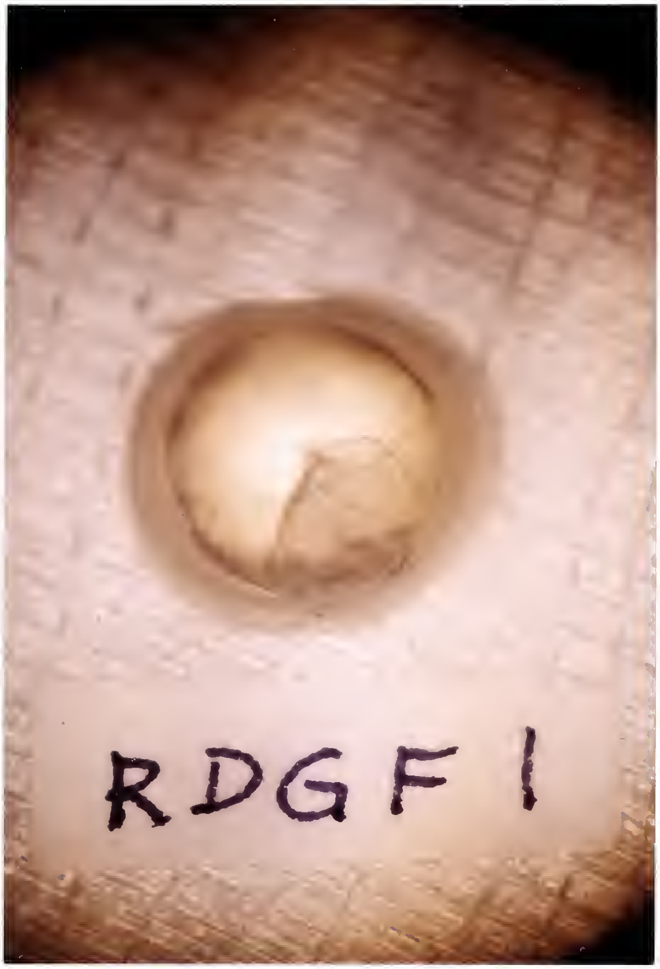


Fig. 8   Photograph showing lens treated with RDGF 71X. Note membranous outgrowth from wound area.

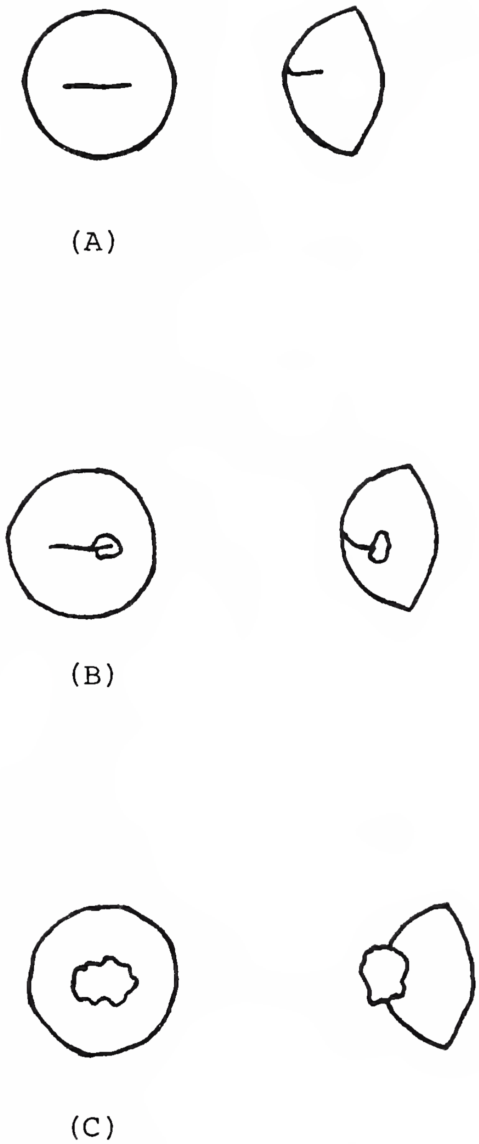


Fig. 9 Anterio-posterior and lateral views of lens lesions. (A) Appearance immediately after lesion. (B) Control lenses, with wound margins well delineated. (C) Lenses treated with RDGF. Note growth of membranous mass from site of injury.

In the control eyes, the wound margins were open and gaped. The surgical incision went through the capsule and subcapsular lens fibers, approximately 2mm deep, in the posterior pole. A part of the capsule had dehisced from the subcapsular fibers and was freely movable from the lens. This capsular material looked like a membrane with ragged edges right at the margins of the wound. There was little opacification of the lens, with some swelling of the remainder of the posterior capsule. Anteriorly the lens was intact. Both control lenses looked very similar; the only difference being the size in length of the wound and the amount of posterior capsule that had detached from the remaining lens fibers.

The two lenses in which a linear lesion was made in the posterior capsule and RDGF 71X was injected showed several differences in appearance from the controls. The lenses showed minimal opacification or swelling. The wound edges grew out of the posterior surface forming a ragged, irregular membrane which grew out approximately 2mm above the lens. It was difficult to make out the wound edges in this, tumor-like mass. Both lenses looked very similar, and different from the two control groups.

D. Histopathologic observations of retinal lesions

One micron thick plastic serial sections made from the retinal lesions were observed under the light microscope. Representative slides from each of the experimental days and conditions were chosen to be reviewed.

The following is the consensus of observations of these representative lesions:

DAY 1

Control

Wounds in the retina, going as deep as the RPE and disrupting Bruch's membrane were studied. No vitreous was seen implanted in the wounds. There were red blood cells, polymorphonuclear cells, and fibrin in the vitreous and scattered over the inner aspects of the wound. Swelling was appreciable in all layers but was more marked in the inner half of the retina. At the retinal hole, the edges rolled inward probably secondary to the edema. The inner limiting membrane was gone from the area of the wound. A defect was seen in the nerve fiber layer. There was swelling of the ganglion cells. There was a dropout of bipolar cells, and those present showed early degenerative changes. The predominant cell type seen in the wound area were the nuclei of the photoreceptors. At the margins of the wound these looked disorganized and almost all layers of the retina had some of these cells present. Some of their nuclei looked pyknotic. The photoreceptors in the wound area were destroyed. There was some detachment with serous infiltrates between the photoreceptors and the RPE. Bruch's membrane appeared broken. The RPE showed little activity, most cells being flat and in close apposition to Bruch's membrane. Some pigment granules were seen drawn almost to the surface

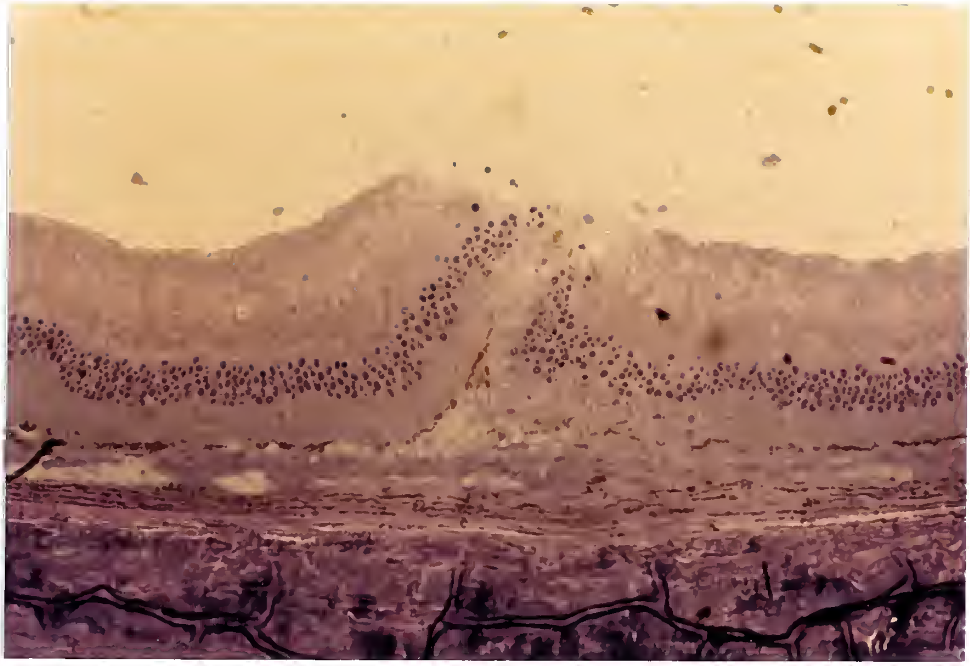


Fig. 10 Photograph showing a representative lesion one day after the injection of HEPES buffer. For description of lesion, refer to pages 48, 50 in the text. (x160, methylene blue, azure II).

of the wound. The cells in the choroid showed no evidence of changes in morphology or proliferation. Very little inflammatory activity was appreciable in the wound area except for a few PMN's present in the vitreous. Sections from different wound areas showed similar changes. In the areas of the retina peripheral to the lesions, no major changes from the normal morphology could be appreciated.

RDGF 71X

Wounds going as deep as the retinal pigment epithelium were observed. The wound margins looked ragged and there was swelling of all the inner layers of the retina. There was no appreciable detachment from the RPE except at the site of the trauma. Red blood cells were observed scattered in the wound area and very few in the preretinal surface and vitreous. Fibrin-like material was observed in the vitreous surface. There was no vitreous incarceration in the wound. The inner limiting membrane was missing at the site of the trauma. There was marked disruption of the nerve fiber layer. There was total loss of the ganglion cell layer, although some were present in the peripheral retina. There was loss of bipolar cells and their position was filled by vacuoles and some remained as empty spaces. The nuclei of the photoreceptor cells, red blood cells, and RPE granules were the predominant constituents of the wound. Some photoreceptor nuclei appeared pyknotic with marked clumping of the chromatin.

The inner segments of the photoreceptors looked normal but the outer segments looked degenerated at the margins of the wound. In the area of the wound there was detachment and movement of RPE towards the surface of the wound, most of these cells contained prominent pigment granules. Some of the RPE cells had lost their flat appearance and now looked round and phagocytic with intracellular pigment granules and cellular debris. There were no cells resembling fibroblasts in the wound area although one of the observers noted some fibrin deposition close to the inner surface of the wound. Bruch's membrane appeared to be broken. The cells in the choroid under the wound showed changes in some of the wounds. They became 3 to 4 cell layers thick and became larger and elongated. Peripheral areas to the wound showed minor changes, primarily in the inner half of the retina where vacuolations were seen in the ganglion and bipolar cell layers with dropout of both of these cell types. It was the general impression of all the observers that slides of Day 1 with the RDGF 71X showed more reparative cellular activity, especially by RPE cells and choroid than the slides observed from Day 1 control.

NGF

A wound in which one of its margins swung out into the vitreous was observed. The lesion went as deep as the RPE. The wound margins were irregular and much cellular disorganization was observed in the inner layers of retina.

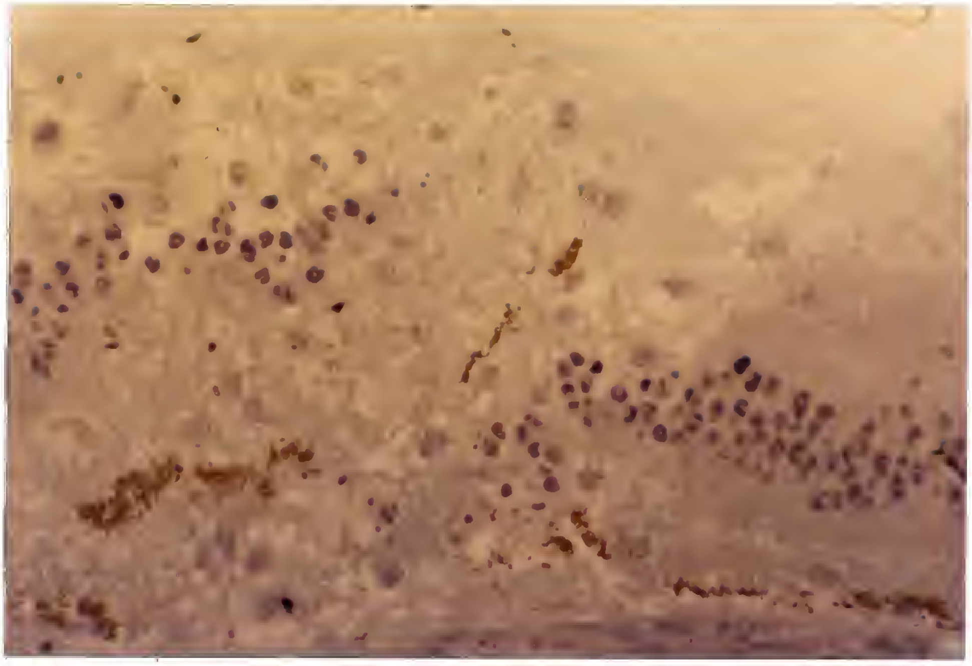


Fig. 11 Photograph of lesion at day one after injection of RDGF 71X. There is cellular disorganization at all levels. Photoreceptor nuclei show pyknosis. This is detachment and movement of the RPE towards the inner surface. For a more detailed description see pages 50, 51. (x400, methylene blue, azure II).

There were no cellular infiltrates or fibrin in the vitreous nor was there vitreous incarceration in the wound. The inner limiting membrane was absent from the site of the wound. The few ganglion cells in the wound area showed marked swelling and vacuolation. The nerve fiber layer was disrupted. The inner layers of the retina showed loss of cellular elements, being replaced mainly by vacuoles, serous fluid, and few migrating pigmented cells. The outer nuclear layer showed pyknosis and degeneration, appearing worse in the areas of frank detachment. The inner halves of the photoreceptors showed less degeneration than the outer halves. There were areas of depigmentation of the RPE close to the wound and some cells containing pigment granules were observed in the inner layers of the retina coming out through the wound. Bruch's membrane appeared intact. The choroid appeared several cell layers thick. The peripheral retina showed little morphologic changes from the control.

RDGF 7.1X

Wounds disrupting all layers of the retina were observed. There was fibrin filling in the empty spaces in the retina left by the wound. The inner edges of the wound were torn by the mechanical trauma and the internal limiting membrane was missing. A few red blood cells were present on the surface of the retina and in the wound cavity close to the base. A fibrin matrix was seen to fill in the gap left by the trauma, and this

matrix appeared to form a scaffold for red blood cells, pigment epithelial macrophages and cellular debris. Few ganglion cells were seen and these appeared to be degenerating with marked swelling and vacuolation. The nerve fiber layer was missing at the site of injury and in the peripheral retina, there was marked vacuolation in this layer. The bipolar cell layer was destroyed at the wound cavity but showed healthy looking cells in all other areas. The outer nuclear layer showed pyknotic nuclei and areas of disorganization close to the wound margin. The photoreceptors were degenerated in the site of the lesion where the retina was detached from the RPE with subretinal pockets of sero-fibrinous fluid. The RPE cells looked very reactive with migration of cells into the inner aspects of the retina, apparently phagocytising the outer segments of some of the photoreceptors. Some cells had lost their pigment granules. The RPE in some of the areas adjacent to the wound appeared to be rounded with more pigment granules than RPE cells in the peripheral areas. Bruch's membrane appeared to be broken and some red blood cells seemed to be coming from the choroid. There was disruption of the choroid by the trauma, with bleeding and disruption of cells. Cells in the choroid close to the wound appeared thin, elongated, and one to two cell layers thick.

Thus, the different wounds and experimental conditions from day one showed more similarities than differences.

The extent of the trauma was comparable between experimental groups and, therefore, the histologic findings at the site of the injury, its margins, and the peripheral retina were very similar.

The inflammatory response in all wounds was much less than expected. Although red blood cells were present in all wounds, the invasion of tissues by white cells was attenuated.

The cells close to the areas of disruption by the injury showed signs of degeneration. There was swelling of the cytoplasm and vacuolation in most cells in the inner half of the retina. The outer nuclear layers close to the margins of the wounds showed clumping of the chromatin, nuclear shrinking and pyknosis. These changes were markedly accentuated in wounds where there was detachment from the RPE. In the areas of the retina peripheral to the wounds, not many changes were noted except in the inner layers, mainly the nerve fiber and ganglion cell layers, where vacuolation was occasionally seen.

The wound area in the two experimental conditions in which the retinoblastoma derived growth factor was injected showed more fibrin deposition. Also, the RPE seems to invade more of the wound area in these two conditions. Some of these cells had changed their morphology, showing phagocytosis of the outer segments of the degenerating photoreceptors and of cellular debris from the inner nuclear layer at the margins of the wound. It is

conceivable that the fibrin seen in these wounds was deposited by the metaplastic RPE cells (Frayer, 1966). But, under these experimental conditions, we can not rule out the possibility of its origin from the vascular elements of the choroid, particularly in wounds where Bruch's membrane was disrupted. No mitotic figures were observed in any of the sections from day one.

DAY 4

Control

All lesions were observed to penetrate into the superficial layers of the choroid. The wound cavity was filled with an exudate, many fibroblasts, and pigmented macrophages. No cells were observed in the vitreous. The wound margins were round and smooth, but elevated secondary to the marked cellular infiltrate. The inner limiting membrane was missing from the wound area. The nerve fiber layer had a defect at the site of the wound. The inner half of the retina showed marked disruption with loss of ganglion and bipolar cells. The only cells that remained in the area seemed to be of glial origin, possibly the foot processes of Müller cells, and the rest of the area was filled with vacuoles, cell debris, and fibroblasts. The outer nuclear layer showed cystic changes, nuclear pyknosis, dropout of cells and thinning in the areas adjacent to the wound. The photoreceptors showed marked degeneration in both inner and outer layers. The RPE seemed to have undergone fibrous metaplasia since

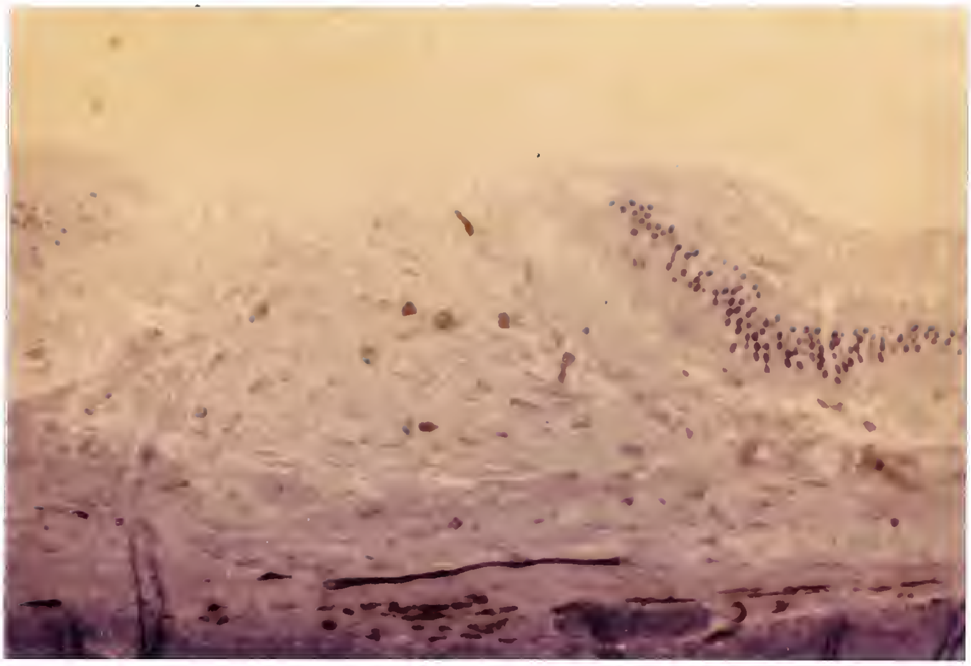


Fig. 12 Photograph of retinal lesion four days after injection with HEPES buffer. Wound cavity is seen to be filled with an exudate, fibroblasts and pigmented macrophages (x400, methylene blue, azure II).

very few pigmented epithelial cells were observed in the wound area yet there were numerous fibroblasts and macrophages containing some pigment granules and cellular debris. In the immediate vicinity of the wound, the RPE was sparse and thin. In more peripheral areas, it was prominent with several cell layers thick and with many pigment granules. There were some large rounded pigment laden macrophages within the subretinal space. Bruch's membrane was broken. The choroid had cells with increased cytoplasmic contents and cells that had fully differentiated into fibroblasts.

RDGF

Sections were studied in which the lesion disrupted all layers of the retina and the superficial choroid. The wound margins gaped inward, with many red blood cells, pigmented macrophages, fibroblasts and exudate filling the wound cavity. Red blood cells and polymorphonuclear leukocytes were present in the vitreous cavity. A few pigmented macrophages had moved into the vitreous surface of the inner retina. Fibrin was present in the wound cavity, forming a scaffold for the other cells in the same area. The retina was detached in the area of the wound and a collection of subretinal fluid was present. The inner limiting membrane was borken off and the ganglion cell layer in the margins of the wound showed much degeneration and replacement by vacuoles and fibrin. The bipolar cells appeared to be better preserved in the

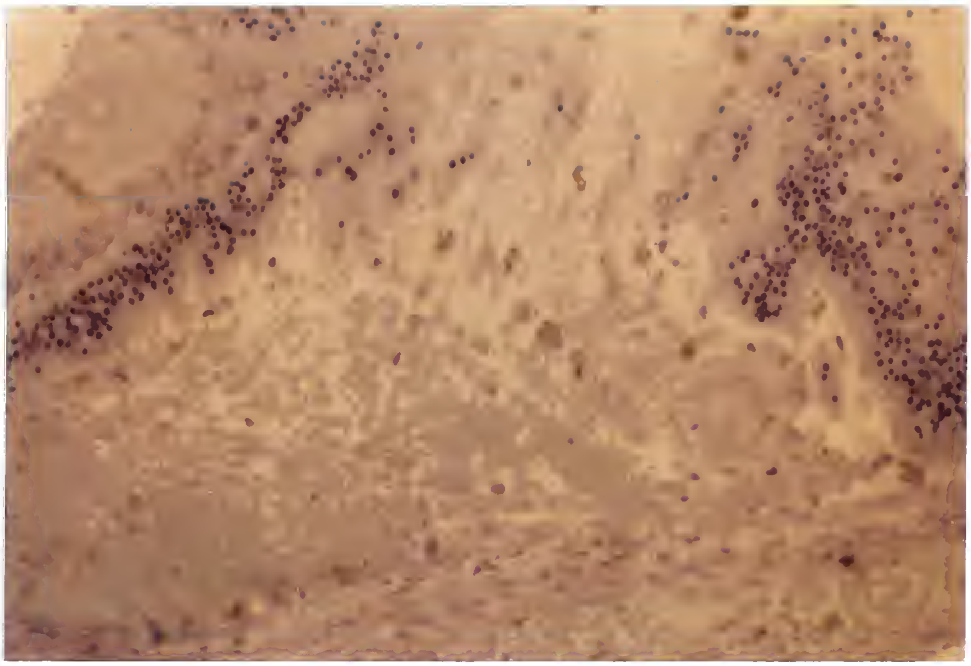


Fig. 13 Photograph of retinal lesion four days after injection of RDGF 71X. Wound extends through all layers of the retina and superficial choroid. Red blood cell debris, fibrin, and pigmented macrophages are seen in the wound cavity. Cells corresponding to the layer of the RPE have lost the pigment granules and appear elongated. (x400, methylene blue, azure II).

periphery of the wound than was observed in the control situation. The inner nuclear layer in the margins of the wound showed marked degenerative changes and the inner and outer segments of the photoreceptors were destroyed. The RPE in the wound area had lost most of the pigment granules, and most of the cells showed changes in their morphology with increased cytoplasm and appeared more elongated. Many of these cells were beginning to look like some of the fibroblasts present in the same areas. Bruch's membrane was broken. The choroidal cells were prominent with increased cytoplasm and, in general, more cells were present here than in the peripheral regions. No major changes were noted in the areas of the retina peripheral to the wounds.

NGF

Wounds studied involved all layers of the retina and penetrated into the choroid, disrupting Bruch's membrane. The wound margins were separated by an infiltrate consisting of red blood cells, pigmented macrophages, fibroblasts and cellular debris. The retina was detached at the wound margins. All cellular layers at the margins of the wound showed marked degenerative cytoplasmic and nuclear changes. The RPE was very reactive at the site of the wound. Most of these cells had lost both their close apposition to Bruch's membrane and the pigment granules. At the margins of the wound, RPE cells were observed to have their long axis oriented in the direction of the wound defect. Bruch's

membrane was broken at the site of injury. Areas of the peripheral retina showed cystic changes in the ganglion cell layer, but otherwise showed no other degenerative changes.

RDGF 7.1X

No lesion comparable to the ones subjected to the other experimental conditions could be obtained. A series was obtained from a wound which showed disorganization of all layers of the retina from the internal limiting membrane, to Bruch's membrane which remained intact. Remnants of photoreceptor nuclei were present in the vitreous and, in fact, these were the predominant cell type in the wound cavity. At the margins, all cellular constituents of the retina showed degenerative changes. The RPE cells directly beneath the injury showed clumping of the pigment granules, but these cells did not show changes in morphology. An active inflammatory response was absent. No changes were observed in the choroid. The peripheral retina showed cystic changes in the inner nuclear and ganglion cell layers. The outer nuclear layer, photoreceptors and RPE did not show any changes. Thus, by day four, most of the lesions showed resorption of red blood cells, less edema and invasion of the wound cavity by fibroblast like cells, pigment macrophages and fibrin deposits. This was a remarkable change from wounds at day one. It appeared as if the reparative cells had used the fibrin deposits as a scaffold to fill in the wound cavity.

There was no observable difference in the separative process between the wounds under the different experimental conditions.

At the margins of the wound, most cells showed the same cytoplasmic and nuclear degenerative changes.

The reactivity of the RPE cells was marked in all the sections. These cells appeared to move from their normal anatomical location to areas within the wound cavity and vitreous. They also changed their morphology, becoming larger and more elongated with their long axis oriented towards the wound cavity.

The retina adjacent, but not involved directly by the trauma, showed little change in most of the sections.

By 7 and 14 days, lesions in the retina under the different experimental conditions were observed to be similar in most of the slides.

At 7 days a defect was noted at the site of injury by the disarrangement of elements in the different layers of the retina. Most lesions contained a fold with its apex oriented towards the inner part of the eye. The outer parts of the retina were in close apposition to Bruch's membrane and most of the subretinal fluid had disappeared. No cells were noted in the vitreous. The retinal edges at the wound margin tapered to form a scar. The wound cavity was completely filled with cellular elements, consisting of a mixture of round, elongated and cuboidal cells; in some of them, the nucleus was

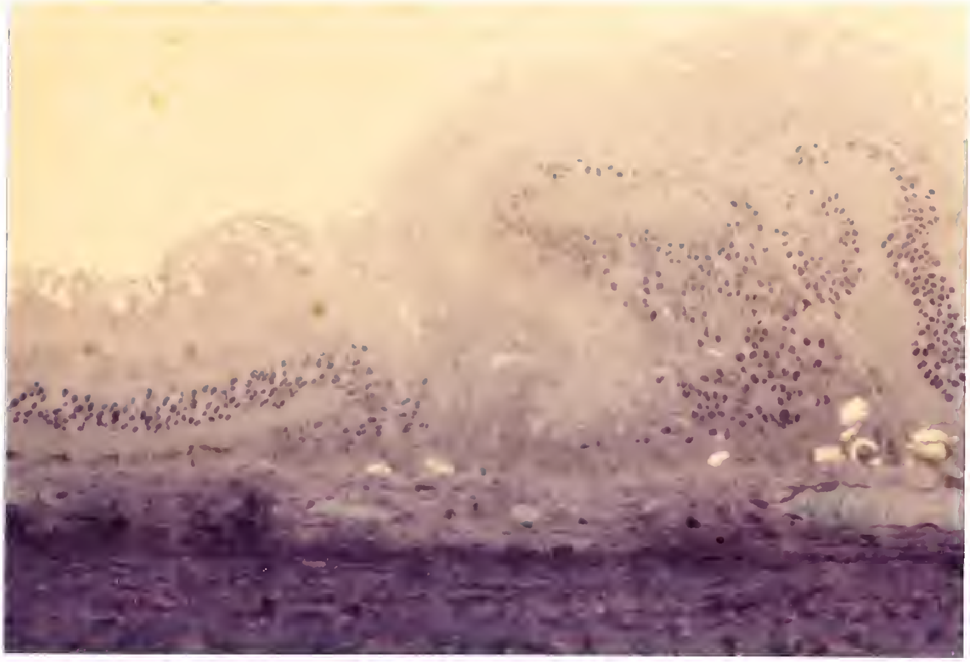


Fig. 14 Photograph of retinal wound after seven days. See pages 62 and 64 for description. (x160, methylene blue, azure II).

indistinguishable. At the wound margins, the prominent elements were the nuclei of the photoreceptors, most of them showing clumping of the chromatin and small pyknotic nuclei. Areas of focal thinning and loss of the outer nuclear layer were observed. Pigment epithelial macrophages had migrated into the spaces between outer segments in areas of retinal damage. The pigment epithelium showed changes in morphology. Many of the cells had lost most or all of their granules.

The parts of the retina peripheral to the wounds showed little changes from their normal morphology.

At 14 days, wounds were seen as folds with the outer nuclear layer in much disarray. These cells together with pigment epithelial cells appeared to fill in the wound cavity. No red blood cells were seen in the wound except intravascularly in the choroid. Most of the exudate was resorbed.

Wound healing progressed from day 7. The margins of the lesion were not completely sealed by scar tissue yet. The outer nuclear layer that was severed from its usual anatomic position appeared degenerated and necrotic. The retinal pigment epithelium, which showed proliferation around the wound sites, formed layers of pigmented, non-pigmented, ovoid and flattened cells at the wound cavity and beneath the damaged retina.

The peripheral retina at 14 days did not show any marked morphologic changes.

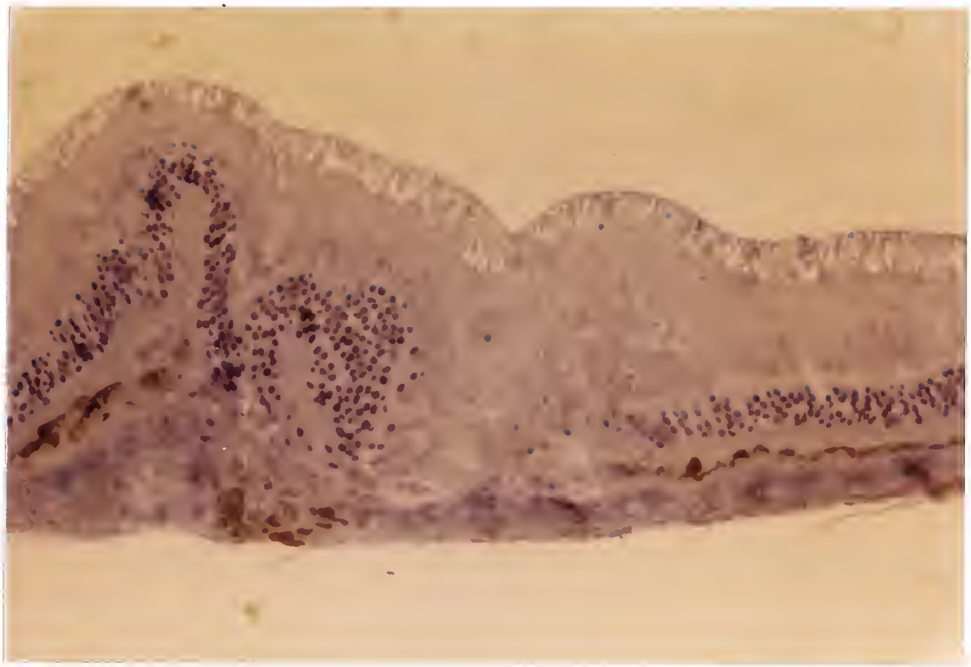


Fig. 15 Photograph of retinal wound fourteen days after the lesion was created. See pages 62 and 64 for description.(X160, methylene blue, azure II).

IV. Discussion

The main objective of this project was to test the stimulatory effects of NGF and RDGF in a living animal and to study the functional consequences of these growth factors on wounds in the lens and retina. The literature provided us with few studies where a growth factor has been characterized with regard to its effects *in vitro* (tissue and organ culture) and *in vivo* (Rich, 1979; Levi-Montalcini, 1960). Observations by other investigators on the mitogenic effect of NGF (Levi-Montalcini, 1960) and of RDGF (Rubin *et al*., 1980, Tarsio *et al*., 1980) in cell cultures raised the question of what would be the *in vivo* consequences of such stimulation.

Gross morphologic evaluations of the lesions in the rabbit lens were suggestive of a different response to no growth factor and to retinoblastoma derived growth factor. When RDGF was injected, lesions in the posterior capsule and subcapsular lens fibers showed a membrane-like growth that extended for approximately 2mm out of the normal lens configuration. It was difficult to identify the wound margins. In the control lenses, the wound margins and subcapsular lens fibers appeared edematous, but no membrane was observed growing. Difficulties were encountered in the preparation of this tissue for histologic evaluation. Scanning electron microscopy was then

attempted. The results of these observations were equivocal for technical reasons, specifically, the preparation of the tissue for electron scanning. The findings, although incomplete, raise the previously asked question of the influences the neural retina may exert in the growth and differentiation of the mature lens (Muthukkaruppan, 1965; Arruti _et_ _al_, 1978). They may also address some of the speculative effects for RDGF in lens development and growth developed from _in_ _vitro_ studies (Tarsio _et_ _al_., 1980). RDGF could be a factor which is normally secreted by the retina for the continued growth of epithelial cells of the mature lens. Another speculative observation is that Y-79 retinoblastoma cells maintained in serum-free medium may be reexpressing cellular genes that are normally repressed in the adult retina. This could result in the synthesis of lens inductive factors responsible for the stimulation of DNA synthesis and proliferation of lens epithelial cells. Our results are insufficient to prove or disprove the above speculations.

Future experiments should attempt to quantify and characterize the histologic appearance of this outgrowth from the wounds in lenses treated with RDGF.

In our model of wounds to the rabbit retina, we were able to observe the different stages of the healing process in the retina, following the same descriptive events of other authors (Hogan & Zimmerman, 1962; Machemer

& Norton, 1968; Yanoff & Fine, 1975; Topping _et al_., 1979; Cleary & Ryan, 1979). We were able to confirm the observations that the rabbit retina is extremely difficult to detach (Machemer, 1968), and also the resistance of the rabbit retina to inflammation and infection following a traumatic lesion (Topping _et al_., 1979). We did not observe healing in the form of plexiform gliosis and accessory gliocytosis (Foos, 1974) possibly because our wounds were not limited to the inner half of the retina, but rather full thickness wounds, most of them through Bruch's membrane. In our lesions we were able to confirm the reactivity of the retinal pigment epithelium (Frayer, 1966; Machemer _et al_., 1978)and its potential for metaplasia into macrophages and fibroblasts (Mandelcorn _et al_., 1975). Our retinal wounds progressed through the healing process much in the same way as skin does, with epithelization and loss of sensory fibers when their nuclei are destroyed (Peacock, 1976).

There were more similarities than differences in the sections of retinal wounds from days 1, 4, 7 and 14 in the control and different experimental conditions. For day one, the lesions in which RDGF 71X and 7.1X was injected, the retinal pigment epithelium appeared more reactive, invading the wound cavity, showing changes in morphology and possibly being responsible for more fibrin deposition. By day 4, all wounds showed approximately the same degree of reactivity by the RPE. No mitotic figures were observed

in the outer nuclear layer. These were qualitative evaluations under light microscopy of selective serial sections, we can not rule out the possibility of quantitative differences that may show a significant effect of these growth factors in the healing of wounds. With more time in the future, we will attempt to do this.

The project reflects the problems encountered in working with an animal model in which many variables may confound the observed results. After several preliminary experiments, we were able to develop our technique of creating the wounds to a high degree of reproducibility. We were unable to control variables that may have existed between the experimental animals, the biologic activity of the growth factors, or the animals response to the injection of a foreign substance. Several unknowns were also present in the experimental model. The half life in the tissues of the growth factor could be assumed from _in vitro_ studies, but the differences between the _in vivo_ and _in vitro_ situations are obvious. The specificity of NGF for any retinal or lens tissue is unknown. Suggestive evidence that RDGF secreted by retinoblastoma cells in culture, may have some specificity for photoreceptor cells comes from the histologic similarities between the tumor cells and the normal photoreceptors (Ts'o _et al._, 1970). Also, rhodopsin has been found in the retinoblastoma tumor cells by antibody assays (Dr. T. Reid, personal communications).

In addition, there is a possibility that the RDGF may contain an inhibitory fraction. This may not be discounted until this substance is further characterized and purified and the stimulation potential of its component parts determined.

V. Conclusions

Reproducible lens and retinal lesions were created in the eye of young Dutch-belted pigmented rabbits. Effects on the healing of these lesions by nerve growth factor and retinoblastoma derived growth factor was evaluated by gross and histologic observations. At two weeks, the lens lesions showed gross morphologic differences between those treated with Hepes buffer and those in which RDGF 71X was injected into the vitreous cavity. Retinal lesions treated with RDGF 71X and RDGF 7.1X showed more reactivity and metaplasia of the RPE and more fibrin deposition by day one than those treated with Hepes buffer and NGF. By days 4, 7 and 14, no appreciable qualitative difference could be observed in the healing of the wounds.

Evaluation of the healing process of the retinal wounds was possible. The retina showed gross traumatic changes due to the lesions in the posterior pole. Full thickness wounds disrupting Bruch's membrane were studied. Near the wound margins, disorganization of the outer and inner segments reflected the physical intensity of the injury. Edema and accumulation of red cells, white cells and RPE proliferation forced inward orientation of the wound margins. Degeneration of inner and outer nuclear layers occurred and this was more marked when the retina was detached. The RPE showed migration into wound cavity

by day one and proliferation, metaplasia, phagocytosis and fibrin deposition as early as day 4. The wound cavity was filled with fibroblasts by day 4 and most of the blood cells were broken down and resorbed. Healing at the wound margins formed smooth and round edges. This process continued through day 14. Photoreceptors and their nuclei showed a marked degree of degeneration at the wound margins and were more prominent in areas where the retina was detached. No mitosis were observed. Changes in the peripheral retina were restricted to losses in the ganglion cell layer and in areas adjacent to wounds, loss of the RPE, which could have possibly migrated to the wound cavity. No vitreous membranes or traction retinal detachment were observed as late as 14 days.

Thus, RDGF seems to influence the formation of a membrane-like structure on a lens wound but we were unable to determine its nature from histology. The effects of RDGF and NGF on a retinal wound were inconclusive although RDGF appeared to stimulate morphologic changes after one day.

VI. References

Abrams, G., Trexler, T., Machemer, R.: An improved method for practice vitrectomy. Arch. Ophthal. 96:521-525 (1978).

Algvere, P., Kock, E.: Experimental fibroplasia in the rabbit vitreous. Albrecht v. Graefes Arch. Klin. Exp. Ophthal. 199:215-222 (1976).

Arruti, C., Courtois, Y.: Morphological changes and growth stimulation of bovine epithelial lens cells by a retinal extract in vitro. Exp. Cell Res. 117:283-292 (1978).

Banerju, S.P., Snyder, S.M., Cuatrecasas, P.: Binding of a nerve growth factor receptor in sympathetic ganglia. Proc. Natl. Acad. Sci., USA, 70:2519-2523 (1973).

Banks, B.E.C., Banthorpe, D.V., Charlwood, K.A.: Distribution and immunology of mammalian nerve growth factor. Nature 246:503-504 (1973).

Barry, D.R.: Pathological changes after trauma. Trans. Ophthal. Soc. U.K. 95:340-345 (1975).

Chanturishvili, P.S. & Stenhouse, D.S.: Regeneration of the lens of the eye. New Scientist 7:1650 (1960).

Cleary, P. & Ryan, S.: Histology of wound, vitreous, and retina in experimental posterior penetrating eye injury in the rhesus monkey. Amer. J. Ophthal. 88:221-231 (1979).

Cleary, P. & Ryan, S.: Experimental posterior penetrating eye injury in the rabbit. II. Histology of wound, vitreous, and retina. Brit. J. Ophthal. 63:312-321 (1979).

Cohen, S.: Purification of a nerve growth promoting protein from the mouse salivary gland and its neuro-cytotoxic antiserum. Proc. Natl. Acad. Sci., USA, 46:302-311 (1960).

Coulombre, A.J.: Experimental embryology of the vertebrate eye. Invest. Ophthal. 4:411-419 (1965).

Coulombre, J.L. & Coulombre, A.J.: Fiber elongation and lens orientation. Science 142:1489-1490

Davidson, M.: The evolution of lens lesions in eye perforations and ruptures. Amer. J. Ophthal. 23:1358 (1940).

Duke-Elder, S.: Diseases of the outer eye. Part 2, Vol. 8, Mosby: St. Louis, Missouri (1965).

Eagling, E.M.: Perforating injuries involving the posterior segment. Trans. Ophthal. Soc. U.K. 95:335-339 (1975).

Fagerholm, P.P., Philipson, B.T.: Experimental traumatic cataract. I. A quantitative microradiographic study. Invest. Ophthal. Vis. Sci. 18/11:1151-1171 (1979).

Fisher, R.F. & Wakely, J.: Changes in lens fibers after damage to the lens capsule. Trans. Ophthal. Soc. U.K. 96:278 (1976).

Fine, B.S. & Yanoff, M.: Ocular histology, a text and atlas. Harper & Row (1972).

Foos, R.: Vitreoretinal juncture - simple epiretinal membranes. Albrecht v. Graefes Arch. Klin. Exp. Ophthal. 189:231-250 (1974).

Foos, R. & Gloor, B.: Vitreoretinal juncture: healing of experimental wounds. Albrecht v. Graefes Arch. Klin. Exp. Ophthal. 196:213-230 (1975).

Francois, J., DeBie, S. & Matton-Van Leuven, M.Th.: Genesis of genetics of retinoblastoma. Jap. J. Ophthal. 22:301-306 (1978).

Frayer, W.: Reactivity of retinal pigment epithelium: an experimental and histopathologic study. Trans. Am. phthal. Soc. 64:586-643 (1966).

Gallie, B.L., Albert, D.M., Wong, J.J.Y., Buyukmihci, N., Puliafito, C.A. Invest. Ophthal. Vis. Sci. 16/3:256-259 (1977).

Gospodarowicz, D. & Moran, J.: Growth factors in mammalian cell culture. Annu. Rev. Biochem. 45:531-558 (1976).

Greene, L.A. & Tischler, A.S.: Establishment of a nonadrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factors. Proc. Natl. Acad. Sci. 73:2424-2428 (1976).

Grierson, I., Johnson, N.: Post-morten changes in the rabbit retina. Acta. Ophthal. 54:529-541 (1976).

Harding, C.V., Feldherr, C., & Srinivasan, B.F.: Structure of the eye. (ed. G.K.Smelser)Academic Press, New York (1961).

Hendry, I.A., Stöckel, K., Thoenen, H., Iversen, L.L.: The retrograde axonal transport of nerve growth factor. Brain Res. 68/1:103-121 (1974).

Hogan, M.J., Zimmerman, L.E.: Ophthalmic pathology, an atlas and textbook. 2nd edition, W.B. Saunders (1962).

Hogue-Angeletti, R.A., Bradshaw, R.A. & Frazier, W.A.: Nerve growth factor: Structure and mechanism of action. In: Advances of Metabolic Disorders, Academic Press, Inc., New York, vol. 8, pp. 285-301 (1975).

Jacobson, M.: Developmental neurobiology, Holt, Rinehart & Winston, New York (1970).

King, J.H. & Wadsworth J.A.C.: An atlas of ophthalmic surgery, 2nd edition, J.B. Lippincott Company (1970).

Lai. Y.: Outward displacement of photoreceptor cells in the normal rat retina (manuscript submitted).

Levi-Montalcini, R. & Hamburger, V.: Selective growth-stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. J. Exp. Zool. 116:321-362 (1951).

Levi-Montalcini, R., Booker, B.: Proc. Natl. Acad. Sci., USA, 46:373-384 (1960).

Levi-Montalcini, R., Calissano, P.: The nerve growth factor. Scientific Amer. 240:69 (1979).

Machemer, R.: Experimental retinal detachment in the owl monkey. II. Histology of the retina and pigment epithelium. Amer. J. Ophthal. 66/3:396-410 (1968).

Machemer, R.: Pathogenesis and classification of massive periretinal proliferation. Brit. J. Ophthal. 62:737-747 (1978).

Machemer, R. & Laqua, H.: Pigment epithelial proliferation in retinal detachment (massive periretinal proliferation) Amer. J. Ophthal. 80:1 (1975).

Machemer, R. & Norton, E.W.D.: Experimental retinal detachment in the owl monkey. Amer. J. Ophthal. 66/3: 388-396 (1968).

Machemer, R., VanHorn, D., Aaberg, T.: Pigment epithelial proliferation in human retinal detachment with massive periretinal proliferation. Amer. J. Ophthal. 85:181-191 (1978).

Mandelcorn, M., Machemer, R. Fineberg, E. & Hersh, St.B.: Proliferation and metaplasia of intra-vitreal retinal pigment epithelium cell autotransplants. Amer. J. Ophthal. 80-227 (1975).

Mueller-Jensen, K., Machemer, R. & Azarnia, R.: Autotransplantation of retinal pigment epithelium in intravitreal diffusion chambers. Amer. J. Ophthal. 80:530 (1975).

Muthukkaruppan, V.: Inductive tissue interaction in the development of the mouse lens in vitro. J. Exp. Zool. 59:269-287

Nevo, Z., Laron, Z.: Growth factors. Amer. J. Dis. Child. 133:419-428

Peacock, E.E., VanWinkle, W., Jr.: Wound repair. 2nd ed., W.B.Saunders Company, Philadelphia (1976).

Pettit, T.H.: A study of lens regeneration in the rabbit. Invest. Ophthal. 2:243 (1963).

Prince, J.H.: The Rabbit in Eye Research. Charles C. Thomas, Springfield (1964).

Rafferty, N.S. & Gossens, W.: Ultrastructural studies of traumatic cataractogenesis: observations of a repair process in mouse lens. Amer. J. Anat. 142:177 (1975).

Reid, T.W., Albert, D.M., Rabson, A.S., Russell, P., Craft, J., Chu, E.W., Tralka, T.S. & Wilcox, J.L.: Characteristics of an established cell line of retinoblastoma. J. Natl. Canc. Inst. 53:347-360 (1974).

Rentsch, F.: Preretinal proliferation of glial cells after mechanical injury of the rabbit retina. Albrecht v. Graefes Arch. Klin. Exp. Ophthal. 188:79-80 (1973).

Rich, L., Weimar, V., Squires, E., Haraguchi, K.: Stimulation of corneal wound healing with mesodermal growth factor. Arch. Ophthal. 97:1326-1330 (1979).

Richardson, K.G., Jarrett, L., Finke, E.H.: Embedding in epoxy resins for ultrathin sectioning in electron microscopy. Stain. Tech. 35:313 (1960).

Riley, M.V.: Ion transport in damaged lenses and by isolated lens epithelium. Exp. Eye Res. 9:28 (1970).

Rubin, N., Tarsio, J., Borthwick, A., Gregerson, D.S. & Reid, T.W.: Identification and characterization of a growth factor secreted by an established cell line of human retinoblastoma maintained in serum-free medium. Vision Res. (in press) (1980).

Rudland, P.S. & DeAsua, L.J.: Action of growth factors in the cell cycle. Biochim. Biophys. Acta. 560:91

Smith, T.R.: Pathologic findings after retina surgery. In: Importance of the vitreous body in retina surgery with special emphasis on reoperations (ed. Ch. L. Schepens) C.V.Mosby, Boston, St. Louis (1960).

Spurr, A.R.: A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruc. Res. 26:31 (1969).

Tarsio, J.F., Rubin, N.A., Russell, P., Gregerson, D.S. & Reid, T.W.: Secretion of a growth promoting activity for Nakano mouse lens epithelial cells by human retinoblastoma cell line (manuscript submitted)(1980).

Topping, T.M., Abrams, G.W., Machemer, R.: Experimental double perforating injury of the posterior segment in rabbit eyes. Arch. Ophthal. 97:735-747 (1979).

Ts'o, M.O.M., Fine, B.S., Zimmerman, L.E.: The nature of retinoblastoma. II Photoreceptor differentiation: An electron microscopic study. Amer. J. Ophthal. 69:350-359 (1970).

VanHorn, D.L., Aaberg, T.M., Machemer, R., Fenze, R.: Glial cell proliferation in human retinal detachment with massive periretinal proliferation. Amer. J. Ophthal. 84/3:383-393 (1977).

Wallow, I.H.L. & Tso, M.O.M.: Repair after xenon arc photocoagulation II. A clinical and light microscopic study of the evolution of retinal lesions in the rhesus monkey. Amer. J. Ophthal. 75:610-626 (1973).

Yamamoto, Y. Growth of lens and ocular environment. Role of neural retina in the growth of mouse lens cells as revealed by an implantation experiment. Dev. Growth Differ. 18:263-278 (1976)

Yanoff, M., Fine, B.S.: Ocular Pathology, A text and atlas. Harper & Row (1975).

Young, M., Saide, J.D., Murphy, R.A.: Molecular size of nerve growth factor in dilute solution. J. Biol. Chem. 251:459-464 (1976).

YALE MEDICAL LIBRARY

Manuscript Theses

Unpublished theses submitted for the Master's and Doctor's degrees and deposited in the Yale Medical Library are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but passages must not be copied without permission of the authors, and without proper credit being given in subsequent written or published work.

This thesis by   has been used by the following persons, whose signatures attest their acceptance of the above restrictions.

NAME AND ADDRESS   DATE